



# **Improving the Handling, Transport and Release of Sterile Male Mosquitoes as Part of an Area-wide Integrated Pest Management Strategy**

Thesis submitted in accordance with the requirements of the  
University of Liverpool for the degree of Doctor of Philosophy

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# Abstract

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Improving the Handling, Transport and Release of Sterile Male Mosquitoes as Part of an Area-wide Integrated Pest Management Strategy

By

Nicole Jean Culbert

The global burden of vector-borne diseases continues to grow year on year. Diseases transmitted by mosquitoes lead to more than 700,000 deaths each year, with malaria alone accounting for almost half a million of the total deaths. Such statistics underline the urgency for alternative complementary control measures. The sterile insect technique (SIT) is one of several genetic control measures routinely used throughout the world to suppress, contain or eradicate various species of agricultural, veterinary or human insect pests. SIT is a technique which has proved successful and sustainable, particularly when deployed as part of an area-wide integrated pest management programme (AW-IPM). A build-up of insecticide resistance coupled with the global spread of species such as *Aedes aegypti* and *Aedes albopictus* has reignited interest in developing mosquito SIT as part of an AW-IPM approach. Significant progress has been made in the last decade towards taking mosquito SIT to the operational level, however, distinct gaps still remain in the literature, especially regarding the post-pupal irradiation stages. The aim of this research thesis was to address some of the key issues where information was lacking, specifically the handling, transport and release of sterile male mosquitoes.

The impact of immobilisation temperature and duration on male mosquito survival was investigated in *Aedes aegypti*, *Aedes albopictus* and *Anopheles arabiensis*, in order to determine a suitable storage and transportation temperature range when conducting releases of sterile male mosquitoes. The effect of compaction during storage was investigated and a maximum tolerable threshold determined. A standardised method to mark male mosquitoes for a small-scale field release was developed and verified in *Aedes aegypti*, *Aedes albopictus* and *Anopheles arabiensis*. A novel flight ability device, which aims to assess male mosquito quality was created and validated for *Aedes aegypti*, *Aedes albopictus* and subsequently modified and verified for *Anopheles arabiensis*. The effect of varying environmental conditions relating to the time of day that sterile male releases could occur was investigated for both male *Anopheles arabiensis* and *Aedes aegypti*. Finally, an adult aerial release device was developed in conjunction with the NGO WeRobotics and as part of a United States Agency for International Development grant. The system was successfully field tested in Brazil via a series of mark-release-recapture studies.

As mosquito SIT nears the operational phase, it is hoped this research is a starting point when addressing some of the outstanding questions related to the handling, transport and release of sterile male mosquitoes.

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## Declaration

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*I declare that this thesis has not been previously submitted for any other degree or professional qualification. This thesis is the result of my own investigations. The results presented in chapters 2 – 8 were obtained in collaboration with others, whose contributions were as follows:*

### Chapter 2:

Conceptualisation and experimental design: NJC<sup>a</sup> and JRLG<sup>b</sup>. Experimental work: NJC. Data interpretation and analysis: NJC. Supervision: JRLG and RSL<sup>c</sup>. Writing original draft: NJC. Writing – review and editing: NJC, RSL, MJBV<sup>d</sup>, ACD<sup>e</sup> and JRLG.

### Chapter 3:

Conceptualisation and experimental design: NJC and JRLG. Experimental work: NJC. Data interpretation and analysis: NJC and JB. Supervision: JRLG. Writing original draft: NJC. Writing – review and editing: NJC, JRLG and JB<sup>f</sup>.

### Chapter 4:

Conceptualisation and experimental design: NJC, FB<sup>g</sup>, AD<sup>h</sup>, GSH<sup>i</sup> and JB. Experimental work: NJC, TW<sup>j</sup> and HY<sup>k</sup>. Data interpretation and analysis: NJC and JB. Supervision: JB. Writing original draft: NJC. Writing – review and editing: NJC, FB, AD, GSH, HY, TW and JB.

### Chapter 5:

Conceptualisation and experimental design: NJC and JB. Experimental work: NJC, NSBS<sup>l</sup>, DDS<sup>m</sup>, SC<sup>n</sup>, TW, HM<sup>o</sup> and WM<sup>p</sup>. Data interpretation and analysis: NJC and JB. Supervision: JB. Writing original draft: NJC. Writing – review and editing: NJC, NSBS, DDS, SC, TW, HM, WM, HY and JB.

## Chapter 6:

Conceptualisation and experimental design: NJC, MK<sup>q</sup>, NV<sup>r</sup> and JRLG. Experimental work: NJC, MK and NV. Data interpretation and analysis: NJC and JB. Supervision: JRLG and JB. Writing original draft: NJC. Writing – review and editing: NJC, MK, NV, MJBV, JRLG and JB.

## Chapter 7:

Conceptualisation and experimental design: NJC, JRLG and WM. Experimental work: NJC, HM, NSBS and WM. Data interpretation and analysis: WM. Supervision: JRLG. Writing original draft: NJC and WM. Writing – review and editing: NJC, HM, NSBS, JRLG, JB and WM.

## Chapter 8:

Conceptualisation and experimental design: JB, NJC, JV<sup>s</sup> and RAH<sup>l</sup>. Experimental work: NJC, MG<sup>u</sup>, AK<sup>v</sup>, JG<sup>w</sup>, TW, GSH, RAH, HY and FB. Data interpretation and analysis: AHD<sup>x</sup> and JB. Supervision: JB and JV. Writing original draft: JB, NJC, AHD, MG, JG and RAH. Writing – review and editing: JB, NJC, AHD, MG, JV, AK, JG, TW, GSH, RAH, HY, FB and MJBV.

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## Dedication

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*I started this journey with you by my side and yet I  
must finish it alone.*

*This one's for you Dad.*

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## List of Abbreviations

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AAT .....	African Animal Trypanosmosis
ATSB .....	Attractive Toxic Sugar Baits
AW-IPM .....	Area Wide–Integrated Pest Management
CDC .....	Centers for Disease Control
CI .....	Cytoplasmic Incompatibility
COPAS .....	Complex Parametric Analyser and Sorter
CRP .....	Coordinated Research Project
FAO .....	Food and Agricultural Organization
GFP .....	Green Fluorescent Protein
GSS .....	Genetic Sexing Strains
HAT .....	Human African Trypanosmosis
IAEA .....	International Atomic Energy Agency
IPCL .....	Insect Pest Control Laboratory
IIT .....	Incompatible Insect Technique
IS .....	Inherited Sterility
KM .....	Kaplan Meier
L:D .....	Light:Dark
L <sub>1</sub> .....	First Instar Larvae
MRC .....	Mass Rearing Cage
MRR .....	Mark Release Recapture
NWS .....	New World Screwworm
OKSIR .....	Okanagan-Kootenay Sterile Insect Release Programme
PARC .....	Plastic Adult Release Containers
PATTEC .....	Pan African Tsetse and Trypanosomiasis Eradication
PMMA .....	Polymethyl methacrylate
QC .....	Quality Control
RH .....	Relative Humidity
RIDL .....	Release of Insects carrying a Dominant Lethal
SIT .....	Sterile Insect Technique
TMRI .....	Tropical Medicine Research Institute
TSS .....	Transgenic Sexing Strains
UN .....	United Nations
USAID .....	United States Agency for International Development
USDA .....	United States Department of Agriculture
WHO .....	World Health Organization

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## Chapter 1

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# Introduction

## 1.1 The World's Deadliest Animal

When asked what the most dangerous animal in the world is, carnivorous, apex predators such as sharks, lions or crocodiles often immediately spring to mind. However, when combined, the average death toll of these animals is estimated to be around 1000 per year (1). What other creature could be, not only more deadly, but also responsible for a staggering 725, 000 deaths worldwide each year (2)? The answer is a considerably smaller creature – the mosquito. Mosquitoes retain their deadly crown year after year because they are major vectors of devastating human diseases, with *Aedes aegypti* considered by some to be the most deadly animal on the planet (3). The role of mosquitoes in spreading disease was first confirmed by Scottish Parasitologist Sir Patrick Manson in 1877 who successfully demonstrated that the *Culex fatigans* (now referred to as *Culex quinquefasciatus*) was the intermediate host of the filarial parasite *Wuchereria bancrofti*, a worm which causes elephantiasis. Known as the founding father of Tropical Medicine, his ground breaking findings in the role of mosquitoes and the spread of disease assisted in the research which confirmed the link between mosquitoes and the malaria parasite by Sir Ronald Ross who went on to win the Nobel Peace Prize for Physiology or Medicine in 1902 (4).

Despite originating from different parts of the world, *Ae. aegypti* (Linnaeus 1762) from Africa and *Aedes albopictus* (Skuse 1894) from the forests of southeast Asia, both species have now become established in every continent except Antarctica. It is postulated that inter-continental trade shipping between the Old and New Worlds by European countries including Spain and Portugal, who would stop off in West African countries including Angola and Senegal to pick up native Africans for the slave trade, allowed *Aedes aegypti* to expand from its native Africa to the Americas around 600

years ago (4, 5). This early trade shipping is also thought to be the culprit for the spread of *Aedes aegypti* to Europe in the early 1800s and throughout the remainder of the century, to Asia, Australia and the Pacific (7). The global spread of *Aedes albopictus* to all inhabited continents of the world, has occurred in only the last 4 decades, arriving in Europe, the Americas and Africa in 1979, 1985 and 1989 respectively (8, 9). The rapid geographical spread of these vectors is attributed to increases in global trade and travel (10). Furthermore, following the process of domestication, both species now breed in artificial containers and feed on human blood, *Aedes aegypti*, almost exclusively (11, 13). The global shipping of tires, a highly prized artificial breeding site for both *Aedes aegypti* and *Aedes albopictus*, has heightened the further spread of both species (14), specifically introducing *Aedes albopictus* into the USA via Houston, Texas in 1985 (9).

Different species of mosquitoes are responsible for spreading various diseases. *Aedes* species including *Aedes aegypti* and *Aedes albopictus* are the vectors of Chikungunya, dengue fever, Yellow fever, Zika and Rift Valley fever. *Anopheles* mosquitoes are vectors of malaria and Lymphatic filariasis, whilst *Culex* species can spread Japanese encephalitis and West Nile fever to name but a few (2). Some diseases such as Eastern equine encephalitis, can be spread by both *Aedes* and *Culex* species. The World Health Organization (WHO) estimates that around half a million deaths attributed to malaria occur each year (15). Additionally, reports suggest that up to 400 million cases of dengue fever are recorded each year with an estimated 3.9 billion people living in an area at risk of contracting dengue (16). Such startling statistics were further exacerbated with the Zika virus outbreak in the Americas in 2015 which has since been linked to Guillain-Barré syndrome and microcephaly in new born babies, with Zika



virus infections reported in a total of 84 countries and territories to date (17). Former WHO leader Margaret Chan at the time, described the spread of the Zika virus as "the price being paid for a massive policy failure that dropped the ball on mosquito control in the 1970s" (18).

## **1.2 A Global Concern**

The global burden of mosquito borne diseases is increasing year on year. An upsurge in population density and in turn globalisation, together with rising global temperatures, brought about by climate change, has resulted in many mosquito species, particularly *Aedes aegypti* and *Aedes albopictus* expanding their natural range (9). This in turn has enabled the geographical range of disease to expand and thus increasing the percentage of the population that live in areas at risk of contracting mosquito borne diseases. In the year 2019, there has been a global surge in the number of dengue fever cases, with the Philippines declaring a national epidemic after a 98% rise in cases during the first 6 months of the year in comparison to 2018 (19).

As it stands, the most common method of conventional pest control is chemical insecticides (20). Larvicides are used to target breeding sites, killing the aquatic stages of the mosquito life cycle, the larvae and pupae. Adulticides are used to target the adults, sprayed both indoors and outdoors or impregnated into bed nets. Insecticides pose a host of negative effects on both the environment and humans, killing non target organisms and contaminating the food chain. Furthermore, years of repeated use has caused resistance to develop within mosquito populations and thus reducing the effectiveness of insecticides. Furthermore, there is a lack of preventative vaccinations available to combat mosquito borne diseases, with the rare exception of Yellow fever.

Additionally, artificial container breeder mosquitoes, notably *Aedes aegypti* and *Aedes albopictus*, mean that current control methods, such as insecticides, have limited effectiveness. With mosquitoes seemingly winning their deadly fight, the race is on to find new, novel or complementary vector control tools to swing the balance back into humanity's favour.

### **1.3 Alternative Vector Control Solutions**

There are many alternative vector control solutions which are currently being developed and trialled in various parts of the world. One relatively simple method is the use of attractive toxic sugar baits (ATSB). ATSBs have shown great promise as a vector control tool in recent years, mass trapping both male and female mosquitoes who are enticed into traps containing a sugar source laced with an insecticide (21, 23). A study in Mali demonstrated that even a single application of an ATSB was able to decimate the local populations of both *Anopheles gambiae* and *Anopheles arabiensis* (24). There are several genetic based approaches which can be seen as promising new vector control tools. An attractive characteristic of genetic based control strategies is that they are environmentally friendly, in complete contrast to the traditional practice of using insecticides as a control method. Their success is mating based, with the vertical transfer of heritable elements, thus making them species specific and posing no risk to non-target organisms (25). One such vector control strategy showing great promise, is the incompatible insect technique (IIT), wherein male mosquitoes are infected with the maternally inherited endosymbiotic bacteria *Wolbachia*. *Wolbachia* can cause a type of sterility within a population of mosquitoes which is referred to as cytoplasmic incompatibility (CI) (26). Embryos produced by uninfected females which were mated with infected males will not develop. Thus,

males infected with the bacteria are sterile when mated with uninfected females and fertile if mated with an infected female. A recent pilot study in Miami, Florida, reported a significant decrease in egg hatch and the number of *Aedes aegypti* in an area where *Wolbachia* infected males were released during a 6 month period in an area of around 170 acres (27).

Another genetic based approach is the Release of Insects carrying a Dominant Lethal (RIDL). RIDL involves inserting a dominant lethal transgene into a strain of the target organism. Expression is artificially repressed to allow the insects to be reared in a laboratory or mass rearing facility (28). By using zygotically active lethal genes, the time of death and the sex of the insect to be killed, can be predetermined (25). Female elimination during the rearing stage, allows batches of pure males carrying a self-limiting gene to be produced. The transgene is transferred to embryos via the RIDL male which results in the death of the zygote during development (29). A field trial in the Cayman Islands successfully demonstrated the feasibility of the RIDL technique in the field (30). A pilot trial which took place in Brazil over a period of one year was further evidence that releasing RIDL males can suppress the local population (29). Following this, releases of RIDL males continued weekly in Brazil for more than 2 years. However, genetic sampling of the local population uncovered that they shared some of the genetic background from the RIDL strain, albeit not the transgenes. This could in theory transform the target population and can occur due to the fact that approximately 3% of the offspring produced from matings between a RIDL male and a wild female survive and subsequently reproduce, forming hybrids (31). It is still unknown if these hybrids will display increased vigor or vectorial competence.

Gene drive is another genetic control technique which could be used as a vector control solution. Although not an entirely new concept, it has seen a surge in interest following the development of CRISPR-cas9, a technique which drastically simplifies the process. Gene drive has the ability to invade an entire population with the release of relatively few insects. As a vector control tool, gene drive could be deployed to modify a target population, for example to reduce their vectorial capacity, or to suppress or even eliminate a population. Gene drive was recently shown to completely suppress a laboratory population of *Anopheles gambiae* (32). The gene *doublesex*, responsible for female development, was targeted. Females with two copies of the gene did not lay eggs and after 8 generations, the mutation has spread throughout the population so that no females were laying eggs, thus, causing the entire population of the malaria carrying mosquito to collapse. Gene drive offers the possibility of removing an entire population or species from a given area, however the implications of doing so are not known and thus population suppression may be a safer approach. One such incidence where gene drive could be deployed to eliminate rather than suppress a population would be against an invasive species, such as *Ae. albopictus*, which is non-native to an area to begin with. The removal of an invasive species should not have any detrimental impact upon the ecosystem or food chain for example, as it shouldn't have been there to begin with. One further concern with the widespread deployment of gene drive is the risk of resistance occurring in the target population via evolution or natural genetic variation (33). The consequences of resistance developing will have major impacts on both the future scientific design of such systems and on the politics of regulating experiments involving gene drive systems (34).

Perhaps the most successful method of genetic based vector control is one which has already stood the test of time against other insect pests but is yet to replicate the same achievements against mosquitoes – autocidal control or more commonly, the sterile insect technique (SIT). SIT is regarded as a target-specific and environmentally friendly method of insect birth control (35). The target insect is colonised, mass reared in large numbers and then subsequently exposed to gamma radiation which causes sterility. Sustained releases of large numbers of sterile insects are carried out to ensure the appropriate sterile to wild overflooding ratio. Following their release, sterile males seek out and mate with wild females, transferring sterile sperm. Although the eggs will become fertilised, the dominant lethal mutations in the transferred sperm mean that embryogenesis cannot occur and in turn no offspring are produced, subsequently decreasing the population of the next generation of the target species. This leads to population suppression or even localised elimination of the target insect if a sufficient number of males are released over a period of time (36, 37).

The aforementioned IIT technique can also be deployed with the irradiation element of SIT incorporated (IIT-SIT), which works as a type of safety net against accidental female release. *Wolbachia* infected males are sterilized using a low irradiation dose, thus any residual females who were not successfully removed during sex separation will be sterile, eliminating the risk of population replacement (38, 39, 40). In a recently published study, IIT-SIT was successfully demonstrated by releasing 200 million sterile males in a region of Guangzhou, China over a 2 year period (2015-2017), resulting in near complete elimination of two field populations of *Aedes albopictus* (41).

## 1.4 The Birth of the Sterile Insect Technique

The practice of using ionizing radiation to induce sterility in insects dates back to the late 1930's and is credited to E. F. Knipling. His theories focused on area-wide integrated pest management (AW-IPM) programmes where SIT could be implemented in both large or small areas containing the target pest for either suppression, prevention or elimination. However he advised that SIT could rarely ever be considered a stand-alone tool and would be most successful when deployed in conjunction with additional complementary vector control measures or following prior suppression of the target insect population (42). In his paper outlining the concept of SIT, Knipling advised that, although the technique would be difficult and costly to implement, even under the most favourable conditions, it may be of use as an eradication tool. For example, highly destructive pests or when preventing the establishment of an invasive pest. He listed 4 key criteria which should be met when considering an insect species as a candidate for SIT. Firstly, it must be possible to mass rear the insect in question in substantial numbers (millions or even billions) in an economic manner. Secondly, the field population of the target insect must be present in low densities to begin with. If this is not the case naturally, preliminary steps to suppress the population, for example using insecticides, should be taken. Thirdly, the process of sterilization should not adversely affect the quality of the male insects. In other words, it must not impede their mating behaviour or negatively affect their survival. Lastly, the released sterile males must be capable of dispersing and seeking out wild female mates (36).

Knipling developed the concept of SIT when researching the New World screwworm (NWS), *Cochliomyia hominivorax* (Coquerel). The NWS causes a parasitic infection known as Myiasis in both humans and animals, however the most devastating effect

is on the latter. It was the first obligate parasite to be reared on an artificial diet and thus the concept of mass rearing was born. Having substantially more insect available to study was key to Knipling's pivotal observation of high sexual aggression in males and the absence of multiple mating in females. He thus postulated that by releasing large numbers of sterile males the wild population would be suppressed or, if the releases continued for long enough, eradicated (Knipling, 1955, 1985). A field trial using SIT against the screwworm undertaken on Sanibel island, 5 km off the Florida coast was deemed successful and was followed by a second trial on the island of Curaçao in 1954, where screwworm eradication was achieved in only 14 weeks. The success of the field trials persuaded the United States Congress to allocate funds and initiate the NWS control program in 1957. The state of Florida was declared screwworm free in 1959 with SIT ultimately validated (43). By mid 1990, NWS eradication had been accomplished in the United States with Mexico, Central America and Panama all achieving screwworm eradication in the years that followed. A permanent barrier where sterile screwworms continue to be released operates over Eastern Panama, thus, ensuring North America remains screwworm free.

A further example of the successful application of SIT against the NWS dates back to 1988 and the introduction of the livestock pest to Libya. The outbreak drew international interest and swift action was undertaken to prevent the pest spreading throughout the African continent. During the height of the eradication campaign, up to 40 million sterile flies were being released each week and by July of 1991, Libya was officially declared screwworm free (44, 45). More recently, Myiasis was detected in Key deer in Florida after decades of being screwworm free. Swift action by the relevant authorities and the rapid deployment of SIT against the NWS prevented the

matter escalating, with eradication achieved after a six month campaign and the release of 188 million flies (46). The ability to eradicate the NWS from North and Central America remain credited as one of, if not the, biggest success story in the history of SIT (44).

### **1.5 Sterile Insect Technique Successes Against Other Diptera**

Currently, SIT is most frequently applied against tephritid fruit flies, extremely destructive pests of fruit and vegetables owing to devastating economical loss and seriously impeding international trade due to strict quarantine regulations (47). One of the most notable plant pests worldwide is the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), due to its crippling damage to fruit and vegetables and the subsequent import bans imposed by pest-free countries (48). Mediterranean fruit fly originated in sub-Saharan Africa and were first detected in the Americas in Brazil shortly after 1900 and its appearance in Costa Rica in 1955 was ultimately what caused its expansion to Guatemala and Mexico by 1977. This realisation prompted the commencement of the first large-scale fruit fly SIT programme as part of an AW-IPM approach in a Memorandum of Understanding between the governments of the USA and Mexico and Guatemala, the formation of the Moscamed programme via collaborative control efforts and the construction of the world's largest mass rearing facility in Metapa, Mexico at this time. The programme aimed to halt any further northward spread of the flies towards the USA, eradicate it from infested areas of Mexico and in the long term, Guatemala and other neighbouring Central American countries (47). For over 4 decades, the programme has successfully acted as a containment barrier for Belize, Mexico and the USA, enabling them to maintain a status of fruit fly free whilst increasing the areas free of pests in Guatemala. This in



turn has facilitated the development of multiple billion-dollar export industries whilst protecting the assets of farmers.

Other SIT programmes have been successfully deployed against the Mexican fruit fly *Anastrepha ludens* (Loew) and the West Indian fruit fly *Anastrepha obliqua* (Macquart). The conception of the Moscafrut facility in Metapa, Mexico in the early 1990s to mass rear *Anastrepha ludens*, aimed to establish fruit fly free areas and strength trade exports. Operations expanded a decade later to include *Anastrepha obliqua*, both of which are still currently mass reared and released which has resulted in 51% of Mexico being declared fruit fly free (49). SIT field trials against the Queensland fruit fly *Bactrocera tryoni* (Froggatt) began in 1962 and since the mid 1990s a fruit fly exclusion zone has been established across key fruit growing areas of the country (47). The melon fly (*Bactrocera cucurbitae*) is a serious agricultural pest throughout Asia, parts of Africa and several Pacific island chains, most notably Hawaii (50). A pilot SIT study was launched on Kume island, Okinawa, Japan in 1972 against the melon fly. Following the successful eradication of the pest, more islands were targeted throughout the archipelago until Okinawa was officially declared melon fly free in 1993.

Tsetse flies (Diptera: Glossinidae) are the cyclical vector of trypanosomes, responsible for Human African Trypanosomiasis (HAT) also referred to as sleeping sickness in humans and African Animal Trypanosomiasis (AAT) or nagana in animals (51). As is the case with mosquito borne diseases, there is a lack of effective vaccines and a build up of resistance of the trypanosomes against available drugs. Thus, vector control remains the most efficient method to manage these diseases (52). Perhaps the biggest

success story of the use of SIT as part of an AW-IPM against Tsetse flies was the total eradication of the species *Glossina austeni* from the island of Unguja, Zanzibar, Tanzania following a release campaign between 1994 and 1997, with the island remaining fly free ever since (53). The success of this campaign prompted the initiation of the Pan African Tsetse and Trypanosomosis Eradication Campaign (PATTEC). Shortly afterwards, several United Nations (UN) Organizations, including the World Health Organization (WHO), the Food and Agricultural Organization (FAO) and the International Atomic Energy Agency (IAEA) consequently passed resolutions to give additional support to the PATTEC initiative (51).

In 2005, the government of Senegal initiated a program called “Projet d’éradication des mouches tsé-tsé dans les Niayes” which aimed to clear an area of 1000 km<sup>2</sup> in the Niayes region, bordering the capital Dakar, of *Glossina palpalis gambiensis* (54, 55). The government opted for an AW-IPM approach with an SIT component following the results of feasibility studies. For the past six years, the Centre International de Recherche-Développement sur l’Elevage en zone Subhumide (CIRDES) in Bobo-Dioulasso, Burkina Faso, the Slovak Academy of Sciences (SAS) in Bratislava, Slovakia, and the FAO/IAEA Insect Pest Control Laboratory (IPCL) in Seibersdorf, Austria have shipped sterile male pupae to Senegal. Following shipment, the adult sterile male flies emerge in an insectary at the Institut Sénégalais de Recherches Agricoles (ISRA) and are subsequently released. Sterile male flies have also been provided by the Insectary of Bobo Dioulasso (IBD) in Bobo Dioulasso, Burkina Faso since 2017 (55).

## **1.6 Sterile Insect Technique Successes Against Lepidoptera**

Lepidoptera species are considered to be amongst the most damaging agricultural pests of both food and fiber crops on a global scale (56). Lepidopteran pests are found in all temperate, tropical and sub-tropical regions of the world and include the codling moth *Cydia pomonella* (Linnaeus), the pink bollworm *Pectinophora gossypiella* (Saunders), the cactus moth *Cactoblastis cactorum* (Berg), the Australian painted apple moth *Teia anartoides* (Walker) and the false codling moth *Thaumatotibia leucotreta* (Meyrick) (57). Following the successful deployment of SIT as part of an AW-IPM against the NWS, investigation into the possibility of using it against Lepidoptera species begun with the codling moth. Lepidoptera are more radiation-resistant than Diptera, with doses above 250 Gy commonly required to induce complete sterility in males (58). A higher radiation dose can greatly reduce an insect's competitiveness and so to counteract this dilemma, a lower, sub-sterilising dose was investigated in codling moth (59). Partially sterile males were mated with wild females, which resulted in a reduced number of F<sub>1</sub> progeny, of which, most were sterile males (60). This phenomenon became a new derivative of SIT aptly named F<sub>1</sub> sterility or inherited sterility (IS). Female Lepidoptera are commonly more susceptible to radiation than males and the radiation dose is often adjusted to fully sterilise females whilst only partially sterilising males. In turn, males are more competitive and live longer. Upon release and copulation with wild females, their subsequent F<sub>1</sub> progeny are mostly sterile males. A major advantage of IS arises from the fact that the sterile F<sub>1</sub> progeny are produced in the field in a natural environment as opposed to an artificial rearing facility. Furthermore, as they have not been subject to radiation, they exhibit a greater competitiveness (57).

The codling moth is a key pest of pome fruit including apples and pears. The development of insecticide resistance within the population and the environmental concerns of prolonged insecticide use has led to over 5 decades of global research concerning SIT/IS against the agricultural pest. A pilot programme was launched in Canada between 1976 and 1978 (61) and by 1992, an operational codling moth AW-IPM programme (OKSIR programme – Okanagan-Kootenay Sterile Insect Release Programme) was initiated in British Columbia, Canada to protect 8000 ha of apple and pear orchards, which still runs to this day (62). More recently, following a pilot study in Hawke's Bay, New Zealand, where sterile codling moths were released by drone over 400 hectares of orchards, a 98% reduction in the wild population was reported (63).

Pink bollworm, *Pectinophora gossypiella* (Saunders) is a major lepidopteran pest of cotton plants, and was first reported in Texas, USA in 1917 after arriving from northeast Mexico (56). It proved to be of particular nuisance in Arizona, southern Californian and the adjacent north western Mexican desert, the main cotton growing areas. SIT has been used as part of an AW-IPM against this major pest since 1968, with around 200 million sterile moths released each week during the cotton season in the San Joaquin valley in southern California alone (64). The AW-IPM campaign includes transgenic cotton expressing insecticidal proteins from *Bacillus thuringiensis* (Bt cotton) coupled with mating disruption with synthetic pheromones (65). Sterile moths are reared in Phoenix, Arizona and shipped to the cotton fields of California for aerial release on a weekly basis.

## 1.7 Historical Applications of the Sterile Insect Technique Against Mosquitoes

After the successful application of SIT against the NWS, efforts were initiated in the 1950s to apply the technique against mosquitoes (42). The first sterile mosquito releases took place in South Florida by the United States Department of Agriculture (USDA) between 1959 and 1960 where around 350 000 male *Anopheles quadrimaculatus* pupae radio-sterilised at 120 Gy were released. The project failed due to a lack of mating between sterile males and wild females, attributed to altered mating behaviour due to the colonisation of the species in the laboratory (66). Various other attempts were made throughout the USA in the years that followed, including against *Aedes aegypti* in Penascola, Florida by the Centers for Disease Control (CDC) between 1960 and 1961 (67) and *Culex tarsalis* in California between 1980 and 1981 (68). The ineffectiveness observed in both such projects was again attributed to incompatible mating behaviour by the released males or a lack of competitiveness derived from the sterilisation process.

The largest and most successful historical implementation of SIT against a mosquito species took place in El Salvador beginning in 1971. At the beginning of the breeding season, male chemosterilized *Anopheles albimanus* were released at Lake Apastapeque, near San Salvador in a 14-15 km<sup>2</sup> pilot site. Pupae were immersed for 60 minutes in an aqueous chemosterilisation solution, bisazir. Using an overflooding ratio of 2:1, around 14 000 sterile males were released daily, preventing the usual rapid increase in vector density normally associated with the malaria transmission season (69, 70, 71). In light of the success of the initial pilot study, a second campaign was initiated in a pilot site of size 150 km<sup>2</sup> in a mountainous region on the Pacific coast,

with the aim to release one million males per day. Concurrently, a male genetic sexing strain (MACHO) was developed, which allowed for female elimination by exposing mosquito eggs to propoxur (*o*-isopropoxyphenyl methylcarbamate), in turn, enhancing production and increasing male competitiveness (72). Despite the success of SIT against *Anopheles albimanus* and the promising field results of the MACHO strain, studies ultimately discontinued due to funds drying up coupled with civil unrest in the country (42).

At the same time as efforts got under way in El Salvador against *Anopheles albimanus*, a joint World Health Organization/Indian Council of Medical Research (WHO/ICMR) experimental programme was initiated in India. The campaign initially targeted *Culex quinquefasciatus*, using the chemosterilising agent thiotepa to induce more than 99% sterility in the males. A mass rearing facility in New Delhi, allowed between 150 000 and 300 000 males to be released daily over a five and a half month period, ensuring an overflooding ratio that was never less than 24:1. Despite a high level of sterility being induced into the local population initially, the sterility level soon began to decrease, due to the immigration of wild females from neighbouring areas (73). Campaigns were also initiated against *Aedes aegypti* and *Anopheles stephensi*, but the programme was ultimately terminated in 1975 due to political reasons (74).

## **1.8 Recent Applications of the Sterile Insect Technique Against Mosquitoes**

Despite a lack of success in early pilot trials following the development of SIT, recent technological and genetic developments have seen the technique pushed firmly back into the lime light. In 2004, the Centro Agricultura Ambiente laboratory (CAA) in

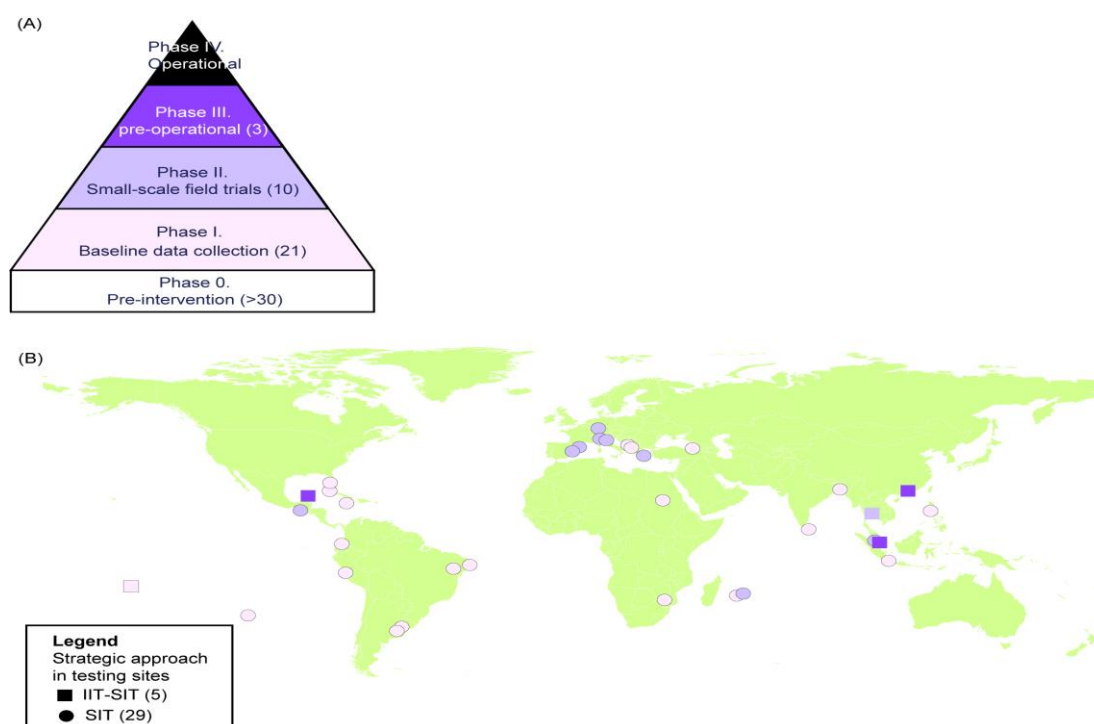
Crevalcore (BO), Italy commenced research addressing the feasibility of introducing a SIT suppression campaign against *Aedes albopictus*. Critical factors, crucial to a successful campaign were explored including gamma ray dosimetry to determine the optimal sterilising dose (75). Additionally, in the summer of 2007, several mark-release-recapture (MRR) studies were conducted in 3 localities in Northern Italy, to determine the survival and dispersal of laboratory reared males (76). Furthermore, mating competitiveness studies were conducted in large enclosures, with males irradiated between 30 and 60 Gy. Results from this study indicated that the 40 Gy sterilising dose recommended in their earlier publication (75) should be revised down to 30 Gy (77). Between 2005 and 2009, five pilot field trials were conducted in three small towns. The induced sterility was estimated by hatching eggs collected weekly from ovitraps situation in both the pilot and control sites. Results indicated that a significant sterility level was induced into the local population when sterile males were released at a rate of between 896 and 1590 males per hectare per week. Furthermore, to obtain a population reduction of between 50 and 90%, the minimum egg sterility value should be 81% (74).

Efforts also commenced against *Anopheles arabiensis* in the Northern State of Sudan in recent years. The Tropical Medicine Research Institute (TMRI) in Khartoum, with the assistance of the IAEA, commenced a feasibility pilot study using SIT in a field site stretching 350 km from Dongola to Merowe, along the banks of the river Nile. Preceded by semi-field studies in large enclosures, males were reared and irradiated in Khartoum and flown 400 km by air to the field site for the pilot study. Results from the study concluded that transporting and releasing sterile males was feasible, at least on a small scale, with emphasis placed on developing and scaling up tools to allow larger numbers to be released in the future (78). A more recent study by the team

showed that sterile male *Anopheles arabiensis* released into the field were able to seek out and participate in existing swarms (79).

In early 2020, a set of guidelines were released by the IAEA detailing a phased conditional approach for mosquito management using SIT within an AW-IPM programme, following recent developments of the technique against mosquitoes (80). It details four phases from preparatory activities to operational deployment as shown in Figure 1. Within a phased conditional approach, support or advancement to the next phase depends on the completion of all of the activities in the previous phase as this minimises the risk of the programme failing. Such guidelines are critical to allow mosquito SIT to transcend from small-scale to large-scale releases in a seamless manner and in order to ensure a standardised method can be deployed on a global scale.

**Figure 1:**





(A) The pyramid symbolises the amount of innovation related to operational research that is needed in the different phases, whereas the volume of activities and investment will, overall, grow in the opposite way. Commitment of the stakeholders will be necessary in all phases, and capacity-building and technology transfer will be specific to each phase. Testing site numbers in each phase are presented in brackets. (B) Distribution of testing sites implementing the sterile insect technique (SIT) against mosquitoes, some of them in combination with the incompatible insect technique (IIT–SIT). Testing site numbers in each strategy are presented in brackets. Phase 0 sites are not shown on the map.

## **1.9 Developing the Mass Rearing Process for Sterile Insects: Pre-irradiation**

In order for a mosquito SIT campaign to reach the operational phase, developing and optimising the mass rearing process was a key starting point. There has been a concerted effort during the past two decades, in particular by the FAO/IAEA laboratories, to meet this goal and fulfil the requests of numerous member states to use the technology as a vector control tool as part of an AW-IPM strategy. Detailed below are some of the breakthrough developments that has seen the concept of mass rearing mosquitoes for a SIT program edge ever closer to the operational level.

### **1.9.1 Larval Rearing**

To bridge the gap from feasibility studies and small-scale pilot trials to a fully operational mosquito mass rearing programme, the development and testing of various pieces of equipment is required. Millions of mosquitoes would potentially have to be produced daily and thus a means of rearing such numbers had to be devised. Within the last decade, a mechanised rearing rack and tray system has been developed, which offers the possibility to hold 200 000 *Anopheles arabiensis* larvae or 1 million *Aedes* larvae, per rack in an area of only 2 m<sup>2</sup> (81, 82). Determining the optimal number of eggs or first instar larvae (L<sub>1</sub>), or in other words, larval density within each rearing tray, in addition to water volume and temperature, are fundamental parameters to

maximise the efficiency of the mass rearing process and to produce high quality adults. A method of quantifying *Anopheles arabiensis* eggs has previously been developed by the FAO/IAEA. By brushing the eggs from an egg paper and drying them, the amount of eggs can be quantified by their weight using the equation 'Weight (mg) = (0.00399 × Number of counted eggs) + 0.536'. This accurate quantification method is rapid and serves to deliver a consistent amount of eggs per rearing tray (83). A recent study investigating the aforementioned parameters for the mass rearing of *Anopheles arabiensis*, concluded that a maximum of 4000 eggs per rearing tray in a volume of 4 litres of water maintained at 27 °C, was the optimal conditions to produce high quality, mass reared adults (84). A further study investigating life history parameters and the mating competitiveness of *Anopheles arabiensis*, reared on a small scale versus a mass rearing scale showed that there was no significant impact on any of the tested parameters between the two scales of rearing (85).

Another recent development advancing mosquito mass rearing towards the operational stage, was the creation of a prototype device that can count L<sub>1</sub> larvae and thus standardise larval density within each rearing tray and in turn reduce labour costs. The current single channel counter has a high accuracy and does not impose any negative impacts upon larval development or survivorship. Increasing the larval input container or creating a multi-channel larval counter could help this tool expand its use in large-scale facilities as mosquito SIT edges closer to the operational phase (86).

Mass rearing at an operational scale would require vast volumes of water, which in arid or seasonally arid countries where fresh water is scarce, may prove to be problematic. The idea of re-using water to rear successive generations of larvae would

be one way of removing this obstacle. A study investigating the re-use of larval water and various ratios of fresh to dirty water, was conducted with *Anopheles arabiensis* as the model species. Results indicated that re-using water did not negatively impact egg hatch rate, larval development time or mortality. However, the quality of the subsequent adult generation was affected (87). In a follow up study, reused larval water was recycled using a water treatment apparatus, a combination of reverse osmosis and ultrafiltration. Astoundingly, adults reared in this recycled dirty water, displayed a significantly higher longevity than both control adults reared in fresh water and those in the untreated dirty water (88). This study serves to demonstrate that if the correct treatment regime is in place, recycled rearing water can be a valuable resource when it comes to mass rearing *Anopheles arabiensis*.

### **1.9.2 Larval Diet**

Larval diet quality is a key driver to produce a high quality of adult males. In recent years, larval diets containing a mixture of bovine liver powder, tuna meal, brewer's yeast and vitamin mix have been developed at the FAO/IAEA laboratories in Austria, for *Anopheles arabiensis*, *Aedes albopictus* and *Aedes aegypti* (89,90). Both diets have been rigorously tested in terms of larval survival, development and adult body size. Recommended daily feeding quantities for each larval instar have also been provided in mass rearing guidelines provided by the FAO/IAEA (91, 92). However, the current recommended diet has encountered availability concerns caused by importation problems of the protein components.

In light of this, research commenced into replacing these components with cheaper and easier to source ingredients and ultimately making the mass rearing process more

cost effective, a key component to the success of any SIT campaign. Several diets were developed and tested with the bovine liver powder component removed and substituted with various edible insects, including black soldier fly (*Hermetia illucens* Linnaeus, 1758), the mealworm beetle (*Tenebrio molitor* Linnaeus, 1758) and the house fly (*Musca domestica* Linnaeus, 1758). The study investigated parameters including mosquito egg hatch, body size, flight ability, longevity and diet cost reduction. Two new diets, wherein the bovine liver powder has been replaced with black soldier fly, are now in the process of becoming officially recommended by the FAO/IAEA for both *Aedes albopictus* and *Aedes aegypti*. Results from this pioneering study indicated that using insects to provide the protein component in a diet to mass rear *Aedes* larvae, is both cost effective and can produce a similar standard of adult mosquito when compared to the current reference IAEA diet (93). Furthermore, depending on the diet composition and species, some life history parameters including female body size, egg production, egg hatch rate and male longevity were even enhanced (94). Future studies aim to adapt the new larval diet for the mass rearing of *Anopheles arabiensis* in the near future.

### **1.9.3 Sex Separation**

The accidental release of females during a mosquito SIT programme, adding to the biting nuisance and potential increase in disease transmission is one that has caused considerable trepidation amongst researchers. It is critical that each batch of mosquitoes released during a campaign are close to 100% males. Thus, sex accurate sex separation has been an area of much focus in recent years and is one of the major bottlenecks that has thus far prevented mosquito SIT reaching the operational phase.

In vectors such as *Aedes aegypti* and *Aedes albopictus*, the use of pupal size for gender prediction can be exploited, due to a size difference between the sexes, with females being larger than their male counterparts in a phenomenon known as sexual dimorphism (95). This natural occurrence, allows the mechanical sorting of pupae, typically via a Fay-Morlan glass plate separator (96) or standard sieves (97). Although highly accurate to some degree, there will always be smaller females and slightly larger males, thus there is an element of female contamination and lost males, in addition to the process being labour intensive. Sex separation in the malaria vector *Anopheles arabiensis* is even more problematic as, there is no sexual dimorphism, thus ruling out any mechanical sorting method at the pupal stage. Additionally, adult separation methods have been investigated by offering mosquitoes bloodmeals spiked with various toxicants, including Ivermectin. In a recent study, Ivermectin (Virbamec®), at a concentration of 7.5 parts per million (ppm), was shown to kill all females within a period of 4 days. The mating efficiency of males caged with females consuming spiked blood was found to be near equal to control males housed with females offered untreated blood (98). There are concerns with this technique however, such as the practical use of such a toxicant in a mass rearing facility in addition to the four day waiting period to eradicate females prior to the release of the males. males caged with females consuming spiked blood were found to be of equal competitiveness as control males housed with females offered untreated blood.

In existing SIT programmes such as that of the Mediterranean fruit fly, genetic sexing strains (GSS), based on classical genetics have been developed, and the application of SIT against this agricultural pest revolutionised. A wild type allele of a white coloured pupae gene was linked to the male determining region via a translocation between an

autosome and the Y-chromosome. This then meant that in all future generations, female pupae were white and male pupae were the usual brown colour (99). Thus, by using optical and mechanical instruments, the pupae could be separated based on colour and enable sterile male-only releases to be possible (100). In subsequent years, a second generation GSS was developed, eliminating all females at an earlier development stage based on a temperature sensitive lethal (tsl) mutation, and in turn decreasing the costs of production (101). Further advancements resulted in the creation of the VIENNA 7 strain which was then replaced with the VIENNA 8 strain (102, 103). More recently, transgenic sexing strains (TSS) have been created, with results indicating performance results equal to that of the VIENNA 8 strain but with a greater cost effectiveness when the production is higher (100). Research is underway with the support of the FAO/IAEA laboratories into the development of a tsl GSS in *Anopheles arabiensis* (104), with the isolation and characterisation of the tsl already achieved. Preliminary analysis suggests that the tsl phenotype is due to a recessive allele located on an autosome (105). Results indicated that the established tsl strain showed similar life history traits to the wild strain, however further research is required before it can be field tested (106).

A GSS was developed for *Anopheles arabiensis* based on a dieldrin resistant mutation and known as ANO IPCL1. Although shown to be reliable for female elimination when dieldrin treatment was added at the larval stage, it did not show the same reliability when used to treat eggs. More recently, the first *Aedes albopictus* GSS was created (Tikok), again by exposing third instar larvae (L<sub>3</sub>) to dieldrin, producing 97.8% males (107). One must remember that, dieldrin, an organochlorine, is known to bioaccumulate in the food chain and thus the prospects of using vast quantities in a

mass rearing facility and the retention of its residues in the released males, mean that that the use of this GSS strain would not be a suitable option for an operational SIT programme (108).

In order to generate a male only population of mosquitoes in a cost-efficient manner, it is necessary to separate the sexes as early as possible during their development. This can be achieved either by killing the females or by removing them based upon sex-specific differential expression of fluorescent marker transgenes such as green fluorescent protein (GFP) (109). Males and females can then be sorted using a complex parametric analyser and sorter (COPAS) flow cytometry machine. A GFP-expressing transgene inserted on the Y chromosome of *Anopheles gambiae* has been isolated. In turn, COPAS-based sorting achieved a near 100% pure male population of the GFP strain (110). Interestingly, the same research group successfully managed to introgress one of their fluorescence-expressing Y chromosomes from *Anopheles gambiae* into *Anopheles arabiensis* (111), which is currently being tested under mass rearing conditions at the IPCL laboratory.

The achievements of some of the major SIT campaigns around the world, including the Mediterranean fruit fly, may not have been possible without the creation of a GSS, or by cost-effective means of removing all female insects during the mass rearing process. Thus, one crucial area where important advances must be made before mosquito SIT application can routinely reach the operational phase and be maintained in a cost-effective manner, is the development of a GSS for each major disease vector.

#### **1.9.4 Irradiation**

Inducing sterility in mosquitoes for the application of SIT is commonly achieved via chemosterilisation, irradiation or more modern biotechnology approaches such as transgenics (28, 35, 112). While research using the chemosterilisation method was abandoned amidst the hazard they presented both to humans and the environment, and the application of transgenics still in its infancy, irradiation as a means of sterilisation currently remains the most practical and reliable method. The irradiation of insects is commonly achieved using gamma rays due to their high energy and penetration. Typically, radioisotopes in the form of Cobalt-60 ( $^{60}\text{Co}$ ) and Caesium-137 ( $^{137}\text{Cs}$ ) are producing the gamma rays used in insect sterilisation, with the former used more frequently as it is easier to manufacture. An additional source of radiation can also be delivered using x-rays. With gamma ray irradiators becoming increasingly difficult to source, transport and reload, x-rays offer a practical alternative (113). A recent study demonstrated that, when using an x-ray irradiator to sterilise *Aedes albopictus*, results were fully comparable with those obtained using a gamma irradiator (114). This result was replicated in a further study published earlier this year, testing the effectiveness of an x-ray irradiator on *Aedes albopictus* at both the pupal and adult stage, irradiated at a dose of 40 Gy (115).

Dosimetry is used to quantify the dose received and a dose response curve must be generated following irradiation with a wide range of doses to pinpoint the optimal dose (116). When determining the optimal dose to administer to an insect, the effects on longevity, sterility and crucially, mating competitiveness must be considered (117). If too low a dose is administered, the insect will not be fully sterile and if the dose is too high, competitiveness will be severely impeded and thus, the released insect will be unable to fulfil their ultimate goal of mating with wild females. Although irradiation



at the pupal stage is easier to undertake, there is evidence that irradiating mosquitoes at the adult stage has less of an impact on their competitiveness (118). Currently, no standardised protocol exists for the adult irradiation of mosquitoes, but it is under investigation at the FAO/IAEA (119). A recent publication has advised on the optimal radiation doses to induce sterility in both *Aedes aegypti* (50 Gy) and *Aedes albopictus* (35 Gy) that do not significantly impact longevity, however, follow up mating competitiveness studies still need to be performed (120). It should be noted that most studies performed to date involve the irradiation of mosquitoes on a small-scale basis. However, in the recently published results of an IIT-SIT mosquito suppression study from China, batches of 65 000 to 70 000 were irradiated inside once canister using an x-ray device (Wolbaki), developed specifically for mosquito irradiation (41). This is by far, the best example of mosquito mass irradiation to date, and paves the way for other programmes around the world. For an operational setting, potentially millions of pupae or adults would need to be irradiated daily and thus, finding methods of achieving this feat whilst offering a uniform dose, is one parameter that requires further research and standardisation before mosquito SIT can become fully operational on a global scale.

### **1.9.5 Mass Rearing Cages**

At both the laboratory and operational level, it is necessary to maintain mosquito colonies in mass rearing cages (MRC) that create a balance between mimicking the species natural habitat and their biological needs, together with high production rates and economic efficiency. MRC must provide adequate space for copulatory flight, whilst offering resting sites, a constant source of sugar and oviposition sites (121). A prototype stainless steel MRC was developed at the FAO/IAEA laboratories for *Aedes*

*albopictus*, with a volume of 100 litres (100 x 100 x 10 cm) and which offered all of the above defined parameters (122). It has since been developed with stepwise changes implemented in recent years to accommodate *Anopheles arabiensis*. Two different versions of the MRC were designed and tested with volumes of either 200 litres (L 200 x H 100 x W 10 cm) or 400 litres (L 200 x H 100 x W 20 cm). No difference in cage productivity was noted following preliminary investigation, albeit the 200 litre MRC is more efficient in terms of space (123). Factors such as the initial pupae loading density have been investigated to determine the optimal number of adult mosquitoes in each cage that generates the highest egg yield, in addition to different blood meal sources from either cattle or pigs. Blood source was found to have no significant impact upon egg production, however, when testing an initial loading density of 15 000 versus 20 000 pupae, the latter was found to have a negative impact upon cage productivity (123). Furthermore, enhancements such as an improved sugar feeding device and the addition of more resting sites and a black cloth cage shroud have been investigated for their effects on adult longevity and egg production, in addition to the frequency of each egg collection (124).

With the aim of reducing the cost of the mass rearing process, a novel plexiglass MRC was developed for *Aedes aegypti* and tested against the current stainless steel version at the IPCL laboratory. Egg production and egg hatch rate were assessed in addition to the validation of an adult-index, wherein mosquito survival rates were determined by counting the number of males and females resting within a 10 x 10 cm square drawn on to the mesh netting of the cage. Egg productivity within the prototype plexiglass cage was of equal measure to the current stainless steel cage, whilst the overall egg hatch rate improved. The longevity of males and females was consistent between both

cages with the adult-index offering a means of gauging adult survival within the cages. Furthermore, the weight of the plexiglass cage is approximately three times less than that of the stainless steel cage, allowing for easier handling and installation. Perhaps the biggest advantage of this novel cage is the cost per unit which has been reduced tenfold in comparison to the stainless steel cage (125).

## **1.10 Mass Rearing Sterile Insects: Post-irradiation**

There remains a lack of research, development and standardisation into the post-irradiation or post-production stages of mosquito mass rearing for the SIT package, despite great advancements in the pre-irradiation stages as outlined in the previous section. Thus, this section will discuss the various post-production stages of mass rearing sterile insects and highlight distinct gaps in the literature and potential stages where information can be gleaned from existing large-scale SIT programmes involving other species of insect.

### **1.10.1 Marking Prior to Release**

Insects are routinely marked prior to being released into the field and subsequently recaptured. Mark-release-recapture (MRR) studies are conducted to gauge information about insect ecology, including dispersal, longevity and to study population density (126). A successful marking method must meet many criteria. It must be cost-effective, easy to apply and long lasting. Furthermore, it must be easily distinguishable, thus that a marked insect can be easily identified from an unmarked one. Lastly, it should not pose any environmental threat nor impose any adverse effects upon the insect itself, including behavioural or reproductive changes or a decrease in longevity (127).

There are a wide range of techniques available concerning the marking of insects, however, it must not be assumed that every method is applicable to each insect species that requires marking. Insects can be marked individually or in groups. Individual marking techniques include using tagging, mutilation methods in addition to paints and inks, where a single dot of colour is applied to an insect with a toothpick for example. Although reliable, individual marking is time consuming and tags are often too heavy for most species but do prove useful for the study of some insects such as honey bees (128).

There are various methods available for marking groups of insects, also referred to as 'mass marking'. One such method is genetic marking, which results in a visible mark. Visible marking commonly targets the body or eye colour and can be induced via naturally occurring mutations or exposure to a mutagen or radiation. This method of marking can be very cost-effective, as once the mutation is discovered, the only associated cost is routine maintenance of the colony (126). Genetic engineering is another technique which is garnering much interest and involves using gene transfer of transposable elements to mass mark insects. An effective system could create a strain of insect that is visibly marked with, for example, GFP, a protein specific to jellyfish. GFP is already being extensively investigated for its potential to mark insects, including mosquitoes (129), yet it is likely to face an uphill battle before it could become widely accepted, due to ethical restraints and public perception.

Radioisotopes were a common way of marking insects in the 1950s through 1970s (130) yet fell out of favour following stricter environmental laws being passed and simpler, more cost-effective methods arising, one of which, was elemental marking.

The element Rubidium (Rb) has been used in studies to investigate the dispersal of aquatic insects such as dragonflies by adding the element to the water in which the larvae live (131). One limitation to the widespread use of using trace elements as a marking source, is that the detection of the elements following exposure can be difficult, time consuming and costly (126).

The most common method of mass marking insects includes the use of paints, dyes and dust. Paints and inks can be applied to large groups of insects, via a spray gun or atomiser for example. It is an inexpensive and rapid way of creating a long-lasting mark. However, it is often a better choice for larger, sturdier insects as the spraying method itself can prove damaging to more fragile insects such as mosquitoes. Dyes have been used as a method of internally marking insects by adding oil-soluble dyes, such as Calco red, to larval diets (132). Although dyes are an inexpensive method of marking insects and one which requires little handling or labour, many dyes have a short retention period or prove harmful to certain species (126). However, one recently emerged marking method involves the use of the fluorescent dye Rhodamine B. The dye, when used to mark mosquitoes, is added to the sugar source and upon feeding, the body and seminal fluid of the males becomes marked. When a male fed with Rhodamine B mates with an unmarked female, it is possible to detect the stained seminal fluid upon the dissection of the spermathecae from the female. When fluoresced, the dye produces a vivid violet-red colour. A recent study has validated the use of the technique in *Aedes aegypti*, both in the laboratory and in a field study (133).

Perhaps the “gold-standard” with regard to the mass marking of mosquitoes, is the use of fluorescent dust. Dusts are available in a wide range of colours, are simple to apply

and do not require the use of sophisticated lights or equipment for their detection. Moreover, it is a method that has stood the test of time, having been used to mark insects for over 70 years (134). Whilst dusts have been shown to be suitable for marking some species of insects (135, 136), there have been studies which report that they are detrimental to others (137, 138). The general consensus within the available literature, is that fluorescent dust *are* a suitable method of mass marking mosquitoes. When comparing the methods of using dust or dye to mark *Anopheles gambiae*, it was reported that neither imposed detrimental effects upon the insects post-marking, as long as marking was carried out within the first 3 days following emergence (139). A study conducted on *Aedes aegypti* using various brands and colours of dusts and paints, in addition to using 4 different methods to apply the mark, reported that the marking method and colour of dust or paint, exerted different effects upon post-marking survival, as well as coverage (127). The above study also noted that using blue fluorescent dust was attributed to reduced survival in *Aedes aegypti*, however, a study involving *Aedes notoscriptus*, reported no negative effects of using blue dust both within their laboratory and MRR studies (140). It appears that although the use of fluorescent dust is a suitable method of marking mosquitoes, the marking technique itself, in addition to the brand and colour of dust can exert different negative effects. Therefore, a gap that could potentially be addressed, would be to create a set of guidelines for marking the main disease-causing vectors such as *Aedes aegypti*, *Aedes albopictus* and *Anopheles arabiensis*.

### **1.10.2 Handling, Packing and Transport for Release**

There remains almost a complete void in the literature when investigating the topic of handling, packing and transporting mosquitoes prior to their release. This is to be

expected, as the operational level has not yet been reached, but one which must be addressed, if the SIT is ever to be an effective tool against mosquito borne diseases. As with every aspect of the mass-rearing process, the handling and transport process can significantly impact the quality of sterile insects if the correct techniques and conditions are not fully optimised. Parameters including temperature, relative humidity (RH) and compaction must be investigated thoroughly to identify suitable environmental conditions for handling, packing and transporting sterile mosquitoes. As large-scale SIT programmes have been fully operational for many decades against fruit flies, tsetse flies and moths, it is here that we must begin the journey to creating the optimal conditions within which to prepare sterile male mosquitoes for either a ground or an aerial release scenario.

Both fruit flies and tsetse flies are perhaps easier to handle, pack and transport prior to a release than mosquitoes are, as this is done so whilst they remain in their pupal phase, which, unlike the mosquito pupal phase, is not aquatic. Fruit fly pupae can be packed into cardboard boxes or plastic bottles whilst tsetse fly pupae are often transported in cartons or petri dishes and transported to emergence or release centres (55, 141). To prevent premature emergence, both fruit fly and tsetse fly pupae are held in a chilled state following irradiation and during transportation. For example, tsetse fly pupae routinely undergo long-distance transportation from rearing facilities in Bratislava, Slovakia and Bobo-Dioulasso, Burkina Faso to Dakar, Senegal, distances of more than 6000 and 2000 km respectively (55, 142). During transportation from the rearing to the emergence centre, which can last up to 84 hours, phase change material is used to maintain a temperature of  $10 \pm 2$  °C inside the shipping container (143). Commonly, sterile fruit fly pupae are shipped at 20 °C, although in some instances, such as when

they are transported between the El Pino rearing facility in Guatemala to Florida, a lower range of 16-18 °C is used due to a higher outside temperature and RH (141).

In SIT programmes against Lepidopteran species such as the pink bollworm and codling moth, shipping is carried out when the insects reach adulthood as it has been shown that the irradiation of adults impedes competitiveness less than during the pupal phase (144). For short transportation durations, such as to conduct a field release experiment, pink bollworm have been maintained between 12 and 18 °C (64). However, frequently sterile moths are required to be transported over longer distances, for example, the weekly shipments of pink bollworm from the rearing facility in Phoenix, Arizona, to the field release sites in California (145). A major SIT campaign against codling moth implemented by the OKSIR Programme in British Columbia, Canada, have reported that insects can be stored at 2 °C for up to 72 hours on occasion prior to release (146, 147). A study which trialled the long-distance shipping of codling moths between Canada and South Africa also reported that there was no significant impact upon longevity or mating ability when transported at between 0 and 1 °C (148). However, when sterile false codling moths were stored between 4 and 6 °C and transported over a period of 12 hours between the Western and Eastern Capes of South Africa, a significant decrease in longevity and flight ability was observed, but fecundity was not affected (149).

In fruit fly SIT programmes, the final step prior to release is emergence. One commonly used method is the plastic adult release containers (PARC) where pupae are volumetrically dispensed into bags (PARCs), small plates (sleeves) or trays (towers). The two most routinely used type of emergence towers are the Guatemala



and the Mexico towers (141). Each packing unit varies by design, resting space and volume, with most large-scale operational fruit fly release programmes now choosing to use the tower method for emergence as it has been shown to impose less of an impact upon longevity and flight ability (150). Emergence rooms are held at around 21 - 23°C/ 70% RH and in total darkness until the flies emerge and are ready to be released. This can range from between 5 to 8 days depending on the species. The flies are also provisioned with food and water prior to release (141).

If the paper bag method is being chosen for an aerial release, the flies emerge inside the bags and maintained at around 20 °C. They are subsequently loaded into an aircraft and ripped open as they are released from the vehicle. This method has been surpassed in most current programmes by the chilled adult release method, which is used in both fruit fly and tsetse fly SIT campaigns. In fruit fly programmes, the emergence towers are chilled at  $3 \pm 1$  °C to immobilise the adults who are then subsequently loaded into large containers to be released by aircraft. The cold chain must be maintained from the point of immobilisation until they exit the release container (141).

Ventilation is another parameter which can severely reduce the quality of sterile flies if not taken into consideration. If the RH is too high, compaction can occur and impede the uniformity of the flies being released. Additionally, too much vibration occurring from the aircraft can exacerbate compaction causing the flies to stick together resulting in clumps being released. In the first aerial release systems which deployed the chilled adult release method, augers were used and subsequently replaced with conveyor belts, but both systems were plagued by compaction issues caused by a rising RH from the metabolic activity of the flies inside the release container (151). Steps were taken to

remedy compaction issues including reducing the humidity in the fly emergence rooms and adding more sophisticated equipment to the release system itself to remove excess humidity and improve cooling. More recently, the Mubarqui company developed a chilled adult release system, compatible with Cessna aircraft and gyrocopters which are currently deployed in all fruit fly releases in Mexico and tsetse releases in Senegal (152).

Of the few studies that have been undertaken regarding mosquito handling and transportation, they have been done so on a small-scale. During a pilot field release of *Anopheles arabiensis* in the Northern State of Sudan, batches of 50 sterile males were transported in paper drinking cups, first by air and then by car to the release site, resulting in minimal mortality of less than 6% (153). The mosquitoes were transported in an active state, with no element of chilling and although promising, for any operational programme, it is highly likely that large volumes of insects will have to undergo chilling prior to and during releases. In a more recent study involving *Aedes aegypti*, storage temperature, compaction level and shipping assays were undertaken to gain insight into the potential to use unmanned aerial systems (UAS) or drones to release sterile male mosquitoes. Results from the study were inconclusive, indicating that higher levels of compaction cause more damage, as one may expect, but that lower compaction rates increased mortality. However, the study did highlight that male *Aedes aegypti* are capable of surviving a wide temperature range of between 7 and 28 °C for up to 24 hours, with little or no effect upon survival (154).

The optimal conditions for storage and transportation vary amongst species of diptera and lepidoptera, with temperature exerting widely different effects on longevity, flight

ability and mating competitiveness as highlighted above. It stands to reason that mosquito species will also react differently to various storage temperatures and conditions; therefore, experiments must be conducted on a species by species basis to define the optimal storage temperature for both short and long-term transportation when any species is being considered for a major SIT campaign.

### **1.10.3 The Release of Sterile Insects**

The release of sterile insects can occur either from the ground or aerially. Historically, in any study involving a sterile component against mosquitoes, release was undertaken from the ground. Moreover, it involved the placing of pots of pupae, allowing emergence to occur in the field. In recent mosquito MRR studies investigating the feasibility of SIT, or the large-scale successful pilot trial conducted in China (41), the release of sterile males has occurred from the ground. Despite their proven success, releasing sterile insects from the air, offers many attractive benefits in comparison to ground releases. Aerially releasing mosquitoes, enables a greater area to be covered faster. This is especially beneficial when considering that most species of mosquitoes generally don't disperse more than a few hundred metres in their lifetime, especially males. Thus, releasing them from the air offers the opportunity to gain a more homogenous dispersal of the sterile males in the target area. Using aerial vehicles would also mean that the cost of the release process, especially so in a fully operational programme, would be greatly reduced in comparison to ground releases. The opportunity to use just one aerial vehicle, as opposed to several ground release vehicles, would also reduce the number of personnel and fuel required. Aerial release also offers the opportunity to reach areas that may be inaccessible by ground such as terrain where there is no vehicular access.

Originally, SIT campaigns involving the release of sterile flies, such as the Mediterranean fruit fly or tsetse fly, were carried out at ground level, either from static fixed points or released by vehicle. Presently, SIT programmes rely on the release of insects by aerial vehicles, which has been shown to be more cost-effective than ground release (155). Aircraft currently used in sterile insect release programmes include various method of fixed-wing vehicles or on occasion, helicopters, when terrain requires a vehicle with better manoeuvrability (156). Some of the most common vehicles involved in aerial release include Cessna airplanes, which serve the large-scale SIT projects in Mexico and the USA, the Beechcraft Baron G58 which is deployed in Guatemala and the Gyrocopter, which is involved in the release of sterile tsetse flies in Senegal (152) and false codling moths in South Africa (157).

For many years, the aerial release of sterile flies was conducted using the paper bag method, where the main focus was on simply getting the insects out of the aircraft. The paper bag served as the release container, in addition to the emergence site for the flies. Although this method was relatively simple, it did have its fair share of problems, including many paper bags collapsing during transport and when being loaded into the aircraft. Furthermore, the paper bag method maintained the adult flies at well above ambient temperature, reducing their subsequent quality and although biodegradable bags were used, it resulted in a great deal of environmental litter (151). In the mid 1970s, a new way of aerial release was developed, involving the release of sterile fruit flies in an immobilised state and is commonly referred to as chilled adult release, as detailed above (158).

There are many factors to be addressed when considering the use of aerial vehicles as part of a sterile insect release campaign. Firstly, each vehicle being considered for the aerial release of sterile insects will have a maximum payload. The payload will ultimately determine the number of insects that can be released in any one flight. If considering a drone for example, the payload may be only a few hundred grams, however a light aircraft such as a Cessna, as used in Mediterranean fruit fly releases, would allow 60 kilograms of payload. Another important consideration is release conditions. Despite aircraft being able to operate under unfavourable weather conditions, the release of insects must only be carried out when conditions are favourable and do not pose a threat to the survival of said insects. Additionally, high winds could create excessive drift and cause the insects to be blown out of the target area. The release speed must also be considered when considering using an aerial vehicle as it determines the flight range and the duration of the flight. It is fundamental to define a suitable release speed to maintain a high level of quality among the released insects. The speed of release must also be accounted for when determining the release rate. Similarly, the release altitude is a critical component of any release. As insects are routinely maintained in an immobile state prior to release, too low an altitude wouldn't allow sufficient time for the insects to wake up prior to landing and too high an altitude could lead to excess drift. Aerial releases are routinely conducted along pre-determined, equally spaced flight paths, also called 'swath'. GPS software is used to determine the flight path prior to each flight and any aerial vehicle involved in a SIT programme must be capable of adhering to the path. If using a light aircraft such as a Cessna, then ground facilities are also required, a runway for take-off and a hanger for storage, in addition to fuel costs and labour costs. The cost of using aerial release

vehicles such as light aircraft, consume a large part of the entire budget for an operational programme, estimated to be as high as 40% (156).

Thus far, the research into using aerial vehicles as part of any mosquito SIT release program has been lacking, and to date, only one paper has been published discussing various options and proposing two unmanned aerial systems (156). There have been several prototype release systems constructed and field tested to be used in conjunction with a small or medium sized drone. However, to date, none of these trials have published their results, leaving a somewhat grey area around the use of drone-based technology as a suitable option for the release of sterile male mosquitoes.

#### **1.10.5 Population Monitoring**

To gain an understanding of the population within a target area for SIT deployment, an accurate estimate of the population is fundamental. Routine population size estimates can help to underpin the ecology of disease vectors, the epidemiology of the diseases they transmit and help to plan for effective control measures. Population monitoring is commonly carried out by inspecting the contents of ovitraps or of adult traps such as BG sentinels. In the context of SIT, population monitoring is crucial with regard to selecting suitable pilot sites, calculating optimal release rates or overflooding ratios, allows a low population season (if any) to be targeted and allows the effectiveness of the vector control technique to be measured.

In order for any programme involving the release of sterile insects to be successful, one of the crucial prerequisites that must be satisfied is that the produced insects are capable of surviving, dispersing and competing and mating with wild counterparts in

the campaign area (159). This can be determined by MRR studies in the field. A pilot site is selected for the MRR, together with an effective trapping method. A means of marking the mosquitoes, such as fluorescent dust, is chosen and the adults released into the pilot site. Traps are then inspected daily for both marked and unmarked adults until the marked adults disappear from the population. A MRR method was first used in the field by researcher Carl Georg Johannes Petersen in 1986 (160), however, the theory behind the method is attributed to Frederick Charles Lincoln, decades before in 1930 (161). The basic statistical equation involved in any MRR is therefore known as the Lincoln Index or the Lincoln-Petersen method. The index itself is relatively simple, however it is based on many assumptions. Due to the low recapture rate of mosquitoes during MRR studies, the Lincoln Index can often overestimate the population size and thus is often modified to compensate for this (162).

Although there have been a vast number of MRR studies carried out in previous years, the majority of them have focused on female dispersal and survival, due to their significance in disease transmission, as opposed to males. However, there are an increasing number of studies being published with regard to MRR trials involving male mosquitoes, with several recent publications presenting the results of MRR studies involving the release of sterile *Aedes albopictus* and with the aim of using the SIT as a vector control tool in the near future. In a publication arising from Mauritius, a series of MRR studies were carried out at different times of the year, in two different habitats, with both irradiated and non-irradiated *Aedes albopictus*, where populations are known to exhibit seasonal population fluctuations. Results indicated that both irradiation and male age at the time of release made no difference to the recapture rate or dispersal. However, habitat type and season, had a significant impact on both (163).

Similarly, a series of MRR studies were carried out during various times of the year, where the local population was displaying its seasonal decline and at both its highest and lowest abundance, at a pilot site on the island of La Reunion. The study aimed to quantify the number of mosquitoes necessary for release in a future SIT campaign in addition to determining the spatial and temporal pattern of releases required, which it did so successfully (164). The pool of literature surrounding male only MRR studies will undoubtedly continue to grow as more and more countries seek to implement SIT campaigns against mosquito disease vectors.

#### **1.10.5 Quality Control**

The quality of mass reared sterile insects is the absolute determining factor in whether a SIT programme is a success or failure. It is imperative that the insects released are of the highest possible quality in order for them to achieve their goal of seeking out and successfully transferring their sterile sperm to wild females. In order to achieve this, sterile males must compete with wild males, males which have not had to endure the pressures of mass rearing, irradiation, immobilisation and transportation to name but a few. It is essential that sterile males have maximal survival and have a similar competitiveness to wild males. Achieving this is a delicate balance as each step of the mass rearing process in addition to the impact from irradiation, handling, transport and release, can all potentially significantly reduce male quality by shortening lifespan or lowering their sexual competitiveness. (165). It is commonplace in any large-scale SIT programme to develop or adhere to an international quality control manual and implement a quality management system (166). Quality control (QC) checks should be made at all stages of the mass rearing process but have been highlighted in this section (post-irradiation) due to the scope of this research thesis.



Means to evaluate the quality of mass reared sterile insects were only developed in the last 40 years and prior to this, insect quality was simply measured on the success or failure of the SIT programme itself (167). The publication of a book detailing ideas for assessing fruit fly quality in the 1970s is generally accredited with developing the subject of sterile insect quality control (168). QC can be divided into three categories, *production* QC which encompasses factors such as diet and rearing equipment, *process* QC, which includes matters such as environmental conditions and irradiation dose, and lastly, *product* QC (insect quality), wherein the actual sterile insect itself is evaluated to determine its effectiveness at completing the job at hand, i.e. survival and mating competitiveness (165). Ultimately, it is insect quality that may be thought of as the most crucial, as the outcomes of the other two, define the overall quality of the sterile insect. Effective quality assurance tests for monitoring and providing feedback at each step of the mass rearing production system, during the handling process and also throughout the eventual release, are vital to ensure a successful programme. There are many life history parameters that require routine inspection via a series of bioassays that can be carried out within a laboratory setting including egg hatch rate, larval development time, pupal size, percentage adult emergence, sex ratio and uniformity of pupal emergence and longevity.

Conducting laboratory bioassays into life history parameters can clarify that the insects being produced are of a high quality, however, they don't fully insure that they will perform as expected once released (169). Semi-field and field bioassays, including mating competitiveness, flight ability, dispersal and longevity must be carried out to ensure that the sterile male insects are of suitable quality (170). One of the most reliable methods of investigating insect quality, is to conduct mating competitiveness

tests, usually in large field cages in semi-natural conditions. This allows the competitiveness index (CI) of the mass-reared sterile males to be established, or in other words, the degree of sterility induced within the population, measured by a reduction in egg hatch rate (171). This is sometimes referred to as the Fried test or Fried Index. However, such tests require vast amounts of space and are extremely time consuming, meaning that they are not carried out as frequently as they should be and in turn, changes in insect quality can go unnoticed.

Flight ability is an elemental trait that influences the quality of sterile insects upon their release into the field and is known to be a direct and reliable marker of insect quality (172). It has also been postulated to be a good proxy of the mating competitiveness of sterile insects (55, 173). Traditionally, flight ability was measured using techniques such as actographs (147, 174) or flight mills (175, 176). However, these techniques were often sophisticated and required specialised training in order to operate the equipment. Furthermore, sample size was limited, meaning multiple tools were required and often, such devices were not suitable for transporting between the laboratory and the field (170). In a range of insects involved in sterile release programmes including fruit flies, tsetse flies and moths, a simple and rapid flight test involving the use of PVC cylinders is now routinely used to determine sterile male flight ability (55,171). Flight cylinders are low-tech, portable and an inexpensive way of determining insect quality. They also allow large numbers of insects to be tested at any given time, as opposed to flight mills, where often a single insect is used.

It stands to reason that flight cylinders could be used to gauge sterile mosquito flight ability and in turn, quality. A recent paper has reported on the first known tool created

to measure the flight capacity of sterile male *Aedes albopictus*. Pupae were placed in individual wells beneath a thin Polymethyl methacrylate (PMMA) tube and upon emergence were tasked with escaping the tubes. Various heights of tubes were tested, with pupae exposed to a range of irradiation doses to impose a decrease in their subsequent quality upon emergence. The outputs of the flight ability test were linked to both survival and mating capacity experiments, which served as quality references. Results indicated that the flight ability test was capable of predicting male quality to some degree. Although tools such as this device are urgently needed to be able to accurately gauge sterile male mosquito quality throughout the mass-rearing process and prior to and following release, the use of a pupal device means that the results are only apparent upon adult emergence. A device that could directly measure the flight ability of adult mosquitoes would allow results to be garnered in less time.

### **1.11 Research Aims and Objectives**

The research presented within this thesis was conducted as part of a five year co-ordinated research project (CRP) investigating mosquito handling, transport, release and male trapping methods, initiated by the Insect Pest Control Sub-programme of the Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture. The CRP includes participants from 21 countries and aims to develop tools and techniques for mosquito SIT in order to make the technique a viable vector control tool. The overall aim of this thesis is to make a significant contribution towards the CRP and mosquito SIT package as it moves ever closer to entering the operational stage. It aims to address distinct gaps in the literature regarding the post-irradiation stage of the mass rearing process (handling, transport and release). The research within this thesis was supported by grants from both the United States Agency for International

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Chapter 2, “*Optimised Conditions for Handling and Transport of male Anopheles arabiensis: Effects of Low Temperature, Compaction and Ventilation on Male Longevity*”, aimed to develop the optimal conditions within which to store and transport male *Anopheles arabiensis* prior to a small-scale release. Males were exposed to a range of low temperatures for 20 minutes to 1) determine if immobilisation occurred 2) how quickly immobilisation occurred and 3) how long it took for flight to resume post immobilisation. Next, males were exposed to a narrower range of immobilisation temperatures for four different durations with survival monitored post-chilling. Volumetric estimations were gathered including the average weight and volume of batches of 1000 males. Additionally, the impact of compaction was investigated in male mosquitoes subject to both short and long periods of immobilisation. Finally, the question of whether immobile adult males can produce metabolic heat was addressed using a thermal image camera.

Chapter 3, “*Investigating the impact of chilling temperature on male Aedes aegypti and Aedes albopictus survival*”, was similar to the first chapter, but in less depth due to time limitations. It involved defining and investigating immobilisation temperatures for male *Aedes aegypti* and *Aedes albopictus*. As before, small batches of adult males were exposed to a range of low temperatures for 20 minutes to investigate when immobilisation occurred. Both species were then further subjected to a 1 of 4

immobilisation temperatures for 1 of 4 durations, with post-exposure survival noted daily.

Chapter 4, “*A Rapid Quality Control Test to Foster the Development of Genetic Control in Mosquitoes*”, details the production and validation of the first tool to be created to measure the quality of adult *Aedes* mosquitoes. Ensuring male mosquitoes are of the highest quality is fundamental to the success of any SIT release campaign. This distinct gap in the literature was within this chapter, which concerns the development and validation of a new flight ability device. Prior to this, there was no easy, inexpensive, rapid and reliable tool available to measure male mosquito quality. Based on flight cylinders, which are routinely used to assess the quality of sterile male fruit flies and tsetse flies, and an early prototype device which aimed to assess the flight ability of *Aedes albopictus* upon emergence, further prototypes flight test devices were constructed and validated for both *Aedes albopictus* and *Aedes aegypti*. Validation tests were carried out by imposing stress treatments (irradiation, chilling temperature and compaction weight) to varying degrees and then assessing flight ability. Survival and mating capacity were measured as they are known to be reliable indicators of mosquito quality, with the results of the flight test modelled against the reference tests to confirm its viability.

Chapter 5, “*A rapid quality control test to foster the development of the sterile insect technique against Anopheles arabiensis*”, builds on the prototype quality control tool described in Chapter 4. Due to the slightly larger male body size of *Anopheles arabiensis* males, a further prototype was constructed to take into consideration this factor. Validation experiments were carried out as described above.

Chapter 6, “*A standardised method of marking male mosquitoes with fluorescent dust*”, presents a standardised guide, with a suggested method, to mark *Anopheles arabiensis*, *Aedes aegypti* and *Aedes albopictus* prior to release for a SIT programme. Despite the available literature suggesting many methods of marking and potential compounds which can be used, conflicting results are presented between studies. The aim of this chapter was to create a simple, inexpensive, long-lasting and non-detrimental method to mark both *Aedes* and *Anopheles* mosquitoes for feasibility studies investigating dispersal and population density via MRR studies or during pilot SIT trials.

Chapter 7, “*Longevity of Mass-reared, Irradiated and Packed Male Anopheles arabiensis and Aedes aegypti Under Simulated Environmental Field Conditions*”, investigates the longevity of sterile male *Anopheles arabiensis* and *Aedes aegypti* subjected to irradiation, immobilisation, packing (compaction) and mimicked environmental conditions upon a simulated release, during either the early morning or the early evening.

Chapter 8, “*Field performance of a mosquito-releasing drone in Brazil*”, was conducted under a USAID grant and in response to the Zika outbreak in the Latin Americas in late 2015. A drone-based aerial release system was developed in partnership with the NGO WeRobotics and field tested in Brazil in April 2018. The project involved a series of rigorous laboratory tests, including release mechanism and environmental conditions testing in addition to a series of MRR trials. The results of the project are presented here.

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## Chapter 2

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# Optimised Conditions for Handling and Transport of male *Anopheles arabiensis*: Effects of Low Temperature, Compaction and Ventilation on Male Longevity

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## Abstract

Developing optimal conditions for handling and transport of sterile male mosquitoes, prior to their release, is critical for the sterile insect technique (SIT) to be successful. No data currently exists for *Anopheles arabiensis* Patton (Diptera: Culicidae) concerning the effects of chilling at different temperatures and for different time lengths on subsequent survival. Additionally, it must be determined whether immobile mosquitoes are capable of producing metabolic heat, the maximum packing density in one release cassette during transport, and their toleration level to compaction. Male *An. arabiensis* were exposed to 2, 4, 6, and 10 °C for 1, 4, 8, or 24 h. Survival was then monitored for 14 days and compared with untreated controls maintained at 25 °C. Short-term (24 h) survival was assessed following immobilisation at 6 °C for 6 h under compacted and non-compacted conditions and compared with non-immobilised controls. The experiment was repeated to assess long-term (1-14 days) survival with varying levels of ventilation. Metabolic heat was assessed in immobilised males (compacted and non-compacted) and compared with males maintained at 28 °C for 2 h. The weight and volume of males were determined to guide the design of the release cassette. Male *An. arabiensis* were maintained at a temperature range of 4-10 °C for 24 h without any significant negative effect on their survival. Compaction did not significantly affect survival; however, it fared better with increased ventilation. Immobile males did not produce any metabolic heat, even when compacted. This study identified initial parameters considered critical to transport sterile male *An. arabiensis* prior to release without any detrimental effect on their survival. Further investigation is required to assess the effects of combining these chosen treatments with irradiation. Additionally, the effect of immobilisation, compaction, and irradiation on the most critical parameter for SIT (male competitiveness) is the subject of future studies.

## Introduction

Mosquito-borne diseases and the subsequent burden placed upon humanity increases annually (1). Insecticide resistance and a lack of vaccines or preventative drugs has increased demand for a sustainable and environmentally friendly solution (2). One such solution is the sterile insect technique (SIT). It is target-specific and can be deployed simultaneously with additional complementary vector management tools as part of an area-wide integrated pest management strategy (AW-IPM) (3). For over 6 decades, SIT has been integrated effectively in programmes against various crop and livestock pests. Successful examples include the eradication of the New World screwworm (*Cochliomyia hominivorax* Cocquerel), Mediterranean fruit fly [*Ceratitis capitata* (Wiedemann)], melon fly [*Bactrocera cucurbitae* (Coquillett)], and the tsetse fly (*Glossina austeni* Newstead) 4-7). Ground releases against the malaria vector *Anopheles arabiensis* Patton (Diptera: Culicidae) are currently being carried out in Sudan with encouraging results (8).

In recent years, significant progress has been made in mosquito mass-rearing methodology (9-10), including the development of cheaper and standardised larval diets (11). The development of an efficient method to separate large numbers of males from females is a major outstanding bottleneck. Despite the existence of genetic sexing strains (GSS), such as ANO IPCL1, its implementation in a large-scale SIT programme is not recommended due to the retention of dieldrin in male mosquitoes (12). Despite promising results from pilot trials, mosquito SIT is still in its infancy. However, we can learn from the experience accrued over decades in SIT programmes involving insects such as the Mediterranean fruit fly (13-14).

Handling, sterilisation, immobilisation, transport, and release are all essential components of an SIT programme. However, each step, if not addressed correctly, could influence male mosquito survival and quality (15). Because the SIT relies on sterile males competing for mates after release, this impact must be minimised to maximise the technique's efficacy. Chilling insects during transport prevents them from moving around and becoming damaged. However, exposing insects to temperatures outside their normal range is known to exert stress upon them. In many instances it reduces insect quality and competitiveness (16). To mitigate any potential negative impact, it is paramount to assess the effects of immobilising such a delicate insect.

Previously, aerial releases of sterile insects involved dosing known volumes of insects into biodegradable release cartons prior to transportation. This approach minimised handling and potential damage (17). More recently, a new continuous release system has been developed to replace the conveyor belt method. It involves releasing chilled adults, such as *Ceratitis capitata* and *Glossina palpalis gambiensis* (Vanderplank), at a defined rate per surface area (18). As release densities are likely to vary between release points within a mosquito SIT programme, this technique may be more desirable. However, in a dosed system, levels of compaction and insect density are low. Thus, a dosed system might be more viable when considering the fragility of mosquitoes. The design of a suitable release device for mosquitoes requires knowledge on (1) the maximum level of compaction of the insects without affecting their survival or performance, and (2) the maximum density of mosquitoes per release container or per unit of volume. Furthermore, the optimum temperature range and the maximum duration to which adult mosquitoes can be exposed must be determined. It has



previously been reported with the Mediterranean fruit fly that the damage inflicted by chilling is directly proportional to the transport time (19).

Additionally, it needs to be established whether immobile adult mosquitoes generate metabolic heat. A temperature increase inside the holding container may result in mosquitoes regaining mobility thus decreasing their quality. This paper aims at providing specific biological information that will facilitate the development of appropriate transport and release technologies required for efficient large-scale field releases of sterile male mosquitoes as part of an integrated vector management strategy with an SIT component.

## **Materials and methods**

### **Mosquito colony rearing**

The Dongola strain of *An. arabiensis* used for this study was sourced originally from the Northern State of Sudan and transferred in 2005 to the Food and Agricultural Organisation/ International Atomic Energy Agency (FAO/IAEA) Insect Pest Control Laboratory (IPCL) in Seibersdorf, Austria. Larvae were reared as was previously described by Maïga et al. (2014) (20) and fed on the IAEA-2 diet developed and described by Damiens et al. (2012) and Yahouédo et al. (2014) (11, 21). Pupae were manually separated from larvae using a cold-water vortex (9) and left to emerge inside 30 × 30 × 30 cm cages (BugDorm, Taipei, Taiwan), with access to a 5% sucrose solution. Adults were maintained in a climate-controlled room at 30 ± 1 °C, 70 ± 10% r.h., and L12:D12 photoperiod, with 1-h periods of simulated dawn and dusk. To determine the temperature and duration threshold for transport, males were separated from females using a mouth aspirator (John W Hock, Gainesville, FL, USA) within 24

h of emergence, after access to a sugar meal. For all other experiments, when more males were required, females were killed by offering fresh bovine blood meals spiked with Ivermectin (Ivomec) as was previously described by Yamada et al. (2013) (22).

### **Determining the temperature and duration thresholds for transport**

Short preliminary experiments were conducted to determine experimental temperatures. Batches of 30 adults were aspirated into small BugDorm cages ( $15 \times 15 \times 15$  cm), placed inside a Sanyo climate chamber (MLR-351H) with a Hero4 GoPro camera for 20 min to test each temperature across the range of 2-14 °C. Four temperatures were chosen for further testing (2, 4, 6, and 10 °C) based on knock out and recovery times. Three batches of 30 adults were immobilised at each temperature for 1, 4, 8, or 24 h; control adults were maintained at  $25 \pm 1$  °C. Immediate mortality was assessed following immobilisation and survival monitored for 14 days, with dead adults removed daily. Survival was not monitored beyond 14 days as survival post-release is not likely to exceed this timeframe.

### **Calculating metabolic heat emissions**

A thermal imager was used (model Ex 5, 10 800 pixels,  $120 \times 90$ ; FLIR Systems, Wilsonville, OR, USA) to measure the amount of metabolic heat generated by immobile adults. Two 9-ml plastic tubes ( $5.6 \times 1.5$  cm) were filled to capacity with immobile adults, with another two filled and gently tapped imposing an element of compaction. One tapped and untapped replicate were left in a cold storage room ( $4 \pm 1$  °C) and the remaining two were exposed to room temperature ( $28 \pm 1$  °C). A thermal image was recorded every 15 min for a period of 2 h. Images were analysed using FLIR Tools software to detect the mean internal tube and external environmental

temperatures.

### **Compaction and survival**

Three 15-ml falcon tubes were filled to the 10-ml mark with immobile adults, and an additional three tubes were filled and gently tapped until the level within each tube had settled at the 3-ml mark. Next, tubes were held upright in a climate chamber at 6 °C for 6 h. Thereafter, each tube was emptied into a cage (30 × 30 × 30 cm), with mortality assessed 1 and 24 h post-immobilisation. Each cage was then placed inside a -20 °C freezer to kill the remaining adults, for counting and survival calculations.

### **Compaction, ventilation, and survival**

Immobile adults were poured into 9-ml plastic tubes, three each with varying degrees of ventilation: normal (tube base removed and each end covered with net, secured with elastic bands), limited (tube opening covered with net and elastic), or none (tube opening covered with plastic paraffin film). All treatments were repeated with adults under compaction, as described above. All tubes were held upright in a climate chamber at 6 °C for 6 h, with three cages of 400 male mosquitoes, maintained at 25 ± 1 °C as controls. Each tube was then emptied into a cage (30 × 30 × 30 cm). Mortality was noted hourly for 4 h, to calculate short-term survival and mortality checks conducted daily for 14 days thereafter.

### **Weight and volume estimation**

Adult male *An. arabiensis* were immobilised at 4 °C for 5 min, counted on ice into batches of 1 000, placed inside a plastic cup, and weighed. The average weight was calculated. They were then transferred to a 50-ml falcon tube to determine their

volume when compacted and non-compacted, as described above.

### **Statistical analysis**

Data were checked for normality with the Shapiro-Wilks Test. Longevity data were examined using Kaplan-Meier (KM) analysis. Log-rank (Mantel Cox) tests compared levels of mortality between treatments and the controls and within treatments. To counteract the problem of multiple comparisons, the Bonferroni correction was used for both experiments.

## **Results**

### **Temperature and duration thresholds for the transport of adults**

GoPro video analysis revealed the minimum temperature required to immobilise adult males to fall somewhere between 11 and 12 °C. Immobilisation did not occur above 12 °C (Table 1). Survival in males exposed to 6 and 10 °C did not differ from control males maintained at 25 °C for any of the tested durations, both 7 and 14 days post-immobilisation (KM:  $P > 0.05$ ). Survival was lower in males exposed to 2 °C for 24 h ( $60.0 \pm 0.01\%$ ) in comparison to those at 4 °C ( $94.4 \pm 0.9\%$ ) and 25 °C ( $92.2 \pm 0.9\%$ ) 7 days after immobilisation (KM:  $\chi^2 = 153.2$ , d.f. = 2,  $P < 0.0001$ ). The same pattern was observed on day 14. Survival was reduced in males exposed to 2 °C for 24 h in comparison with both 4 and 25 °C (KM:  $\chi^2 = 135.9$ , d.f. = 2,  $P < 0.0001$ ) (Table 2).

When survival was compared between durations, no significant differences were observed in males immobilised at 6 or 10 °C. Survival was lower in males exposed to 2 °C for 24 h ( $60.0 \pm 0.01\%$ ) in comparison to those maintained for 1, 4, and 8 h ( $94.4 \pm 1.2$ ,  $87.8 \pm 0.9$ , and  $86.7 \pm 1.0\%$ , respectively), 7 days post-immobilisation (KM:  $\chi^2$

= 172.3, d.f. = 3,  $P < 0.0001$ ). After 14 days, survival in those subjected to 2 °C for 1 h was higher ( $92.2 \pm 1.3\%$ ) than after exposure for 4, 8, or 24 h (KM:  $\chi^2 = 153.6$ , d.f. = 3,  $P < 0.0001$ ) (Figure 1). Additionally, survival in those immobilised at 2 °C for 4 and 8 h ( $80.0 \pm 2.0$  and  $78.9 \pm 1.5\%$ , respectively) was significantly higher when compared to males exposed for 24 h ( $58.3 \pm 0.5\%$ ).

**Table 1:**

Temperature (°C)	Time until knock out (s)	Recovery (s)
14	No knock out	0
13	No knock out	0
12	Partial knock out	0
11	$25 \pm 2.5$	$12 \pm 1.5$
10	$21 \pm 3.4$	$21 \pm 3.5$
9	$19 \pm 1.6$	$25 \pm 1.8$
8	$20 \pm 1.2$	$46 \pm 4.0$
7	$19 \pm 2.6$	$60 \pm 1.6$
6	$22 \pm 1.8$	$95 \pm 4.9$
5	$21 \pm 0.5$	$101 \pm 6.1$
4	$23 \pm 2.3$	$110 \pm 8.7$
3	$16 \pm 3.8$	$120 \pm 6.1$
2	$12 \pm 3.6$	$210 \pm 5.5$

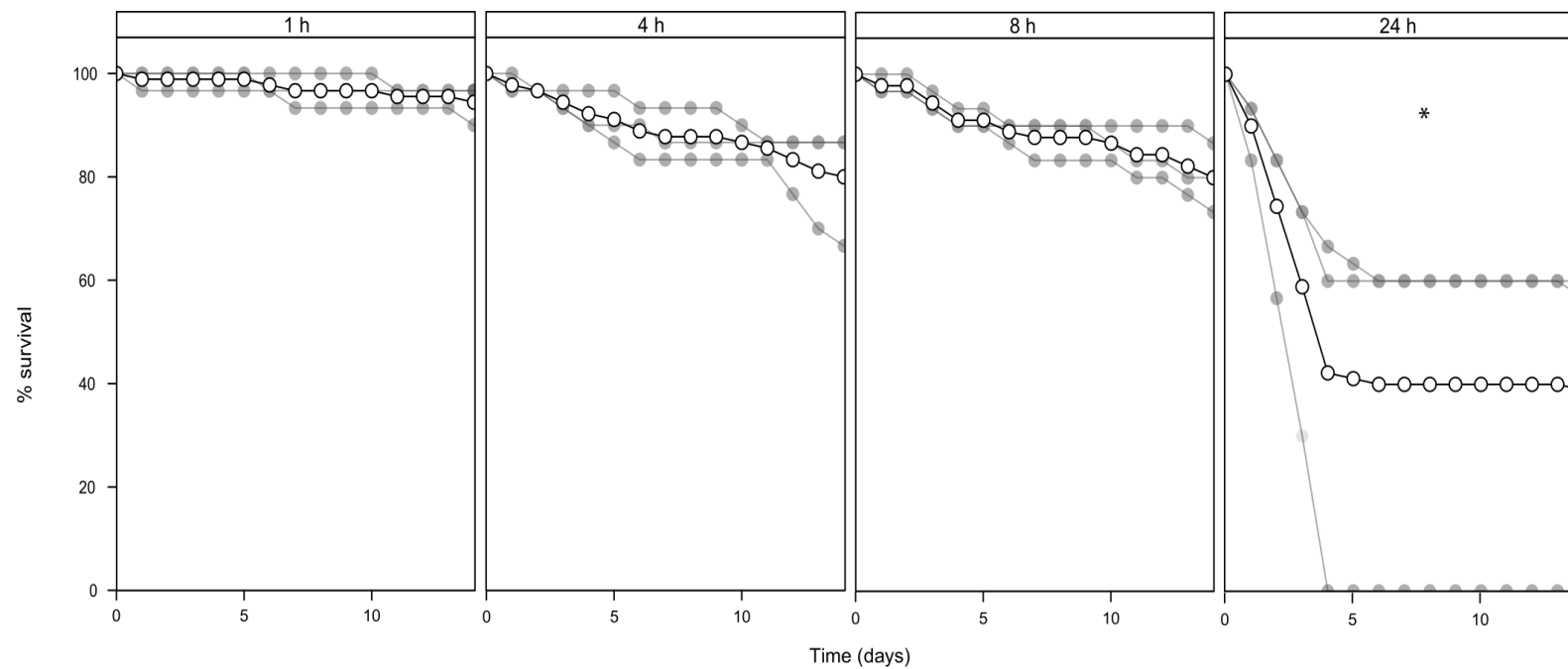
Mean ( $\pm$  standard error, SE) knock out and recovery times (seconds) of adult male *Anopheles arabiensis* following exposure for 20 min to a constant temperature (range 2-14 °C). Time until complete immobilisation and recovery after exposure was noted

**Table 2:**

Duration (h)	Temperature (°C)					
	2	4	26	6	10	25
1	92 ± 1.3	89 ± 0.9	89 ± 0.3	80 ± 0.6	76 ± 1.7	71 ± 1.2
4	80 ± 2.0	89 ± 1.7	91 ± 0.7	80 ± 1.5	73 ± 2.9	73 ± 1.5
8	79 ± 1.5	90 ± 1.2	88 ± 1.5	70 ± 3.2	74 ± 0.9	67 ± 1.5
24	58 ± 0.5	90 ± 0.6	84 ± 0.9	61 ± 1.8	73 ± 1.5	70 ± 2.6

Mean ( $\pm$  standard error, SE) % survival of adult male *Anopheles arabiensis* 14 days post-immobilisation at 2, 4, 6, or 10 °C for 1, 4, 8, or 24 h, compared to controls maintained at 25 and 26 °C.

**Figure 1:**

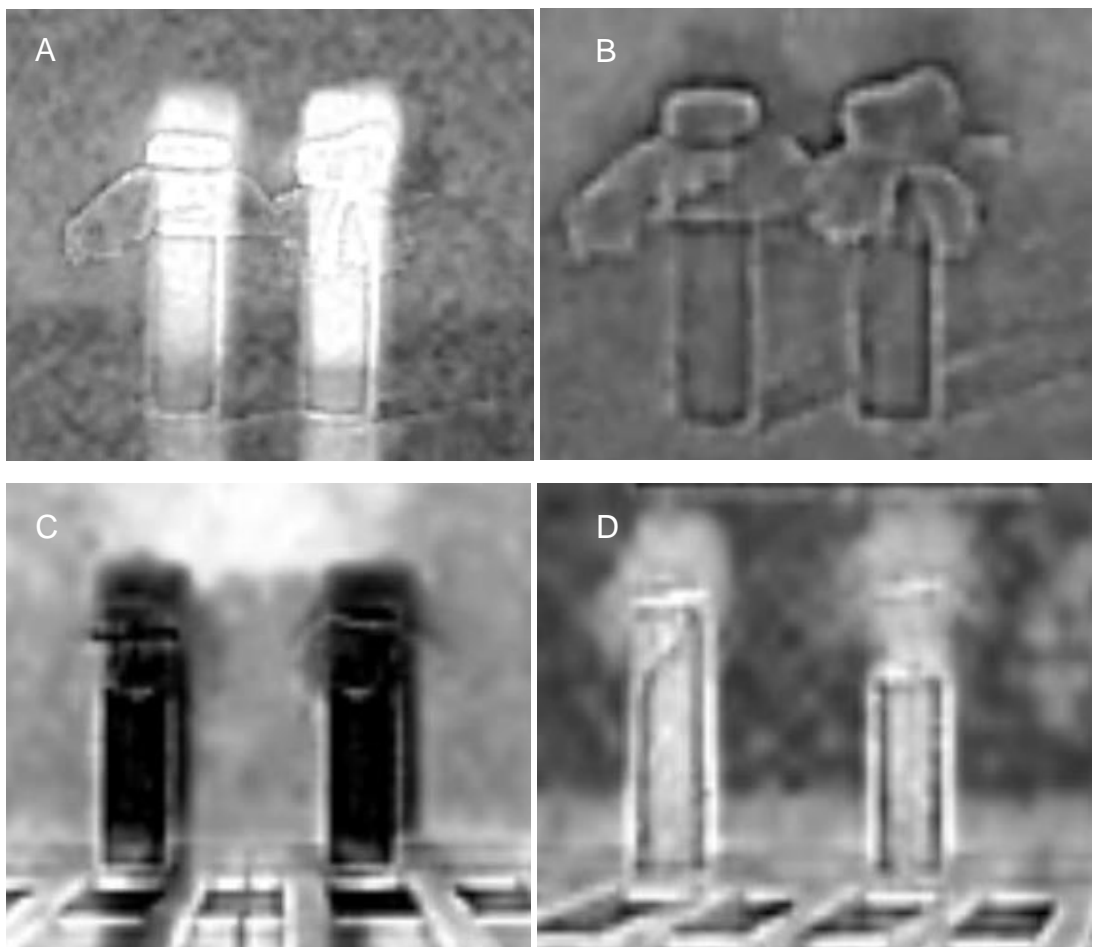


Survival % of adult male *Anopheles arabiensis* for up to 14 days following immobilisation at 2 °C for 1, 4, 8 or 24 h. Mean values are represented by white circles with a black outline. Each series of grey dots represents a repetition. An asterisk denotes a significant difference.

### Metabolic heat emissions

Tubes filled with both compacted and non-compacted males immobilised and held at 4 °C had a higher temperature than the surrounding air in the first 5 min (Figure 2A). After 30 min, the temperature inside each tube varied by less than 0.1 °C and both tubes were now around 0.2 °C cooler than that of the external environment (Figure 2B) and remained so for 24 h (data not shown). Conversely, the tubes filled with immobile males and then moved to a room maintained at 27 °C were initially much cooler than the surrounding air temperature (Figure 2C). However, after 30 min, both tubes were 0.2 °C warmer than the external ambient temperature of the laboratory, again varying by 0.1 °C between compacted and non-compacted adults (Figure 2D).

**Figure 2:**





Metabolic heat generated by (A,B) immobile and (C,D) active male *Anopheles arabiensis*. Adult males at 4 °C after immobilisation for (A) 5 min or (B) 30 min. Active males (C) 5 min and (D) 30 min after transfer to 27 °C following immobilisation. \*White and black colouration represents a warmer and cooler temperature than the surrounding environment respectively.

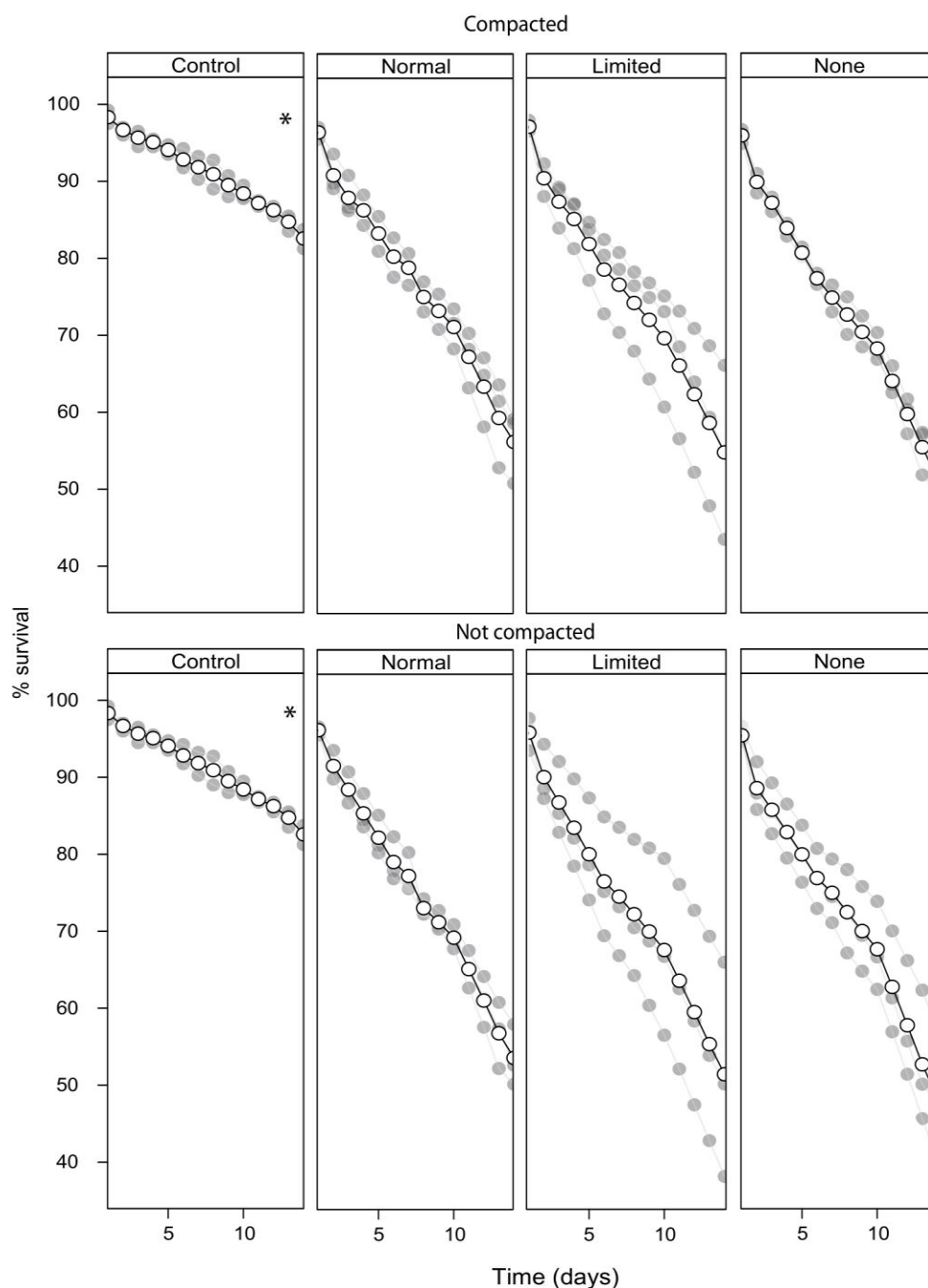
### **Effect of compaction on survival**

No difference in survival after 24 h was observed between tapped ( $95.7 \pm 1.66\%$ ) and untapped ( $96.2 \pm 0.23\%$ ) replicates (KM:  $P > 0.05$ ). Following this result, a long-term study was initiated with results detailed below.

### **Effect of compaction and ventilation on survival**

Short-term survival (1-4 h) was lower in all three treatments in comparison with the control groups for both compacted (KM:  $\chi^2 = 70.32$ ) and non-compacted samples (KM:  $\chi^2 = 75.85$ , both d.f. = 3,  $P < 0.0001$ ). Survival of all non-compacted treatment groups was lower than in the control group on day 14 post-immobilisation (KM:  $\chi^2 = 378.9$ , d.f. = 3,  $P < 0.0001$ ) (Figure 3A). This result was also seen in compacted samples (KM:  $\chi^2 = 438.4$ , d.f. = 3,  $P < 0.0001$ ) (Figure 3B). However, there was no difference in survival between compacted and non-compacted treatment groups (KM:  $P > 0.05$ ) on day 14 post-immobilisation.

**Figure 3:**



Survival % of compacted and non-compacted male *Anopheles arabiensis* subjected to 6 hours of immobilisation at 6 °C with varying degrees of ventilation: normal (tube base removed and each end covered with mesh), limited (tube opening covered with mesh) or none (tube opening covered with plastic paraffin film). Controls were maintained in the lab at  $25 \pm 1$  °C. Mean values are represented by white circles with a black outline. Each series of grey dots represents a repetition. An asterisk represents a significant difference.

### Weight and volume estimation

Batches of 1 000 immobile adult males weighed on average  $1.47 \pm 0.01$  g and had a volume of 12.5 ml, or 7.5 ml if compacted (Table 3).

**Table 3:**

No. males	Weight (g)	Volume (ml)		No. males ml <sup>-1</sup>	
		Compacted	Non-compacted	Compacted	Non-compacted
1000	$1.47 \pm 0.01$	7.5	12.5	133	80
2000	$2.94 \pm 0.02$	14	24	143	83
4000	$5.89 \pm 0.01$	27	46	148	87

Mean ( $\pm$  standard error) weight (g) of 1000, 2000, and 4000 adult male *Anopheles arabiensis* and estimates of the volume (ml) they occupy when compacted or non-compacted (tapped or untapped).

### Discussion

With the aim to improve transport and aerial release methods, we determined key parameters that influence the biological quality of transported sterile male *An. arabiensis*: volume and weight of adult males, and the effects of compaction and ventilation on male survival. Furthermore, a suitable temperature range for transport of male mosquitoes was defined, and we showed that mosquitoes did not emit metabolic heat, whilst held in bulk in an immobile state.

Sterile male tsetse pupae are transported at a temperature of around 8-10 °C (15) to prevent emergence of the male flies during transport. In view of the slow reproductive cycle of tsetse flies, females need to be retained in the colony. There is no GSS available for tsetse flies, but the development time of females is 1-2 days shorter than that of males. After female emergence, males are chilled to block their emergence

during transport. Sterile male mosquitoes are most likely transported as adults. We recommend that they are maintained immobile between 4-10 °C during transportation. Males were not held immobile for longer than 24 h. Thus, it remains to be seen whether they can endure 48 or perhaps 72 h without a significant subsequent impact upon quality. This temperature range is in line with the temperature used to maintain sterile Mediterranean fruit flies (19) and tsetse flies (15) during an aerial release ( $10 \pm 3$  °C). Similarly, the ladybug *Coccinella magnifica* Redtenbacher could be maintained for up to 30 h at 10 °C without any detrimental effect (23). Based on our results, 6 °C may be selected as the optimal transport temperature for male *An. arabiensis*, which would allow a range of ca.  $\pm 3$  °C during the transport period. Another reassuring finding was that we noted no immediate mortality following the process of immobilisation at any of the tested temperatures or chilling durations. We did, however, notice a difference in survival between the two control groups. It should be noted that not all temperatures were tested at the same time due to a lack of climatic chambers. Six and 10 °C were investigated first, followed by 2 and 4 °C. After analysing the data extracted from the data loggers, it was found that there was a difference of 1 °C in ambient temperature between the two groups. The control group that accompanied 6 and 10 °C were, on average, exposed to 25 °C, whereas the second control group was maintained at 26 °C. This difference may have been critical and perhaps explains the difference in survival between the two control groups.

The quality of Mediterranean fruit flies is known to be compromised during transport as a result of compaction (19). The metabolic activity of the flies, coupled with vibration produced by the release vehicle, causes them to aggregate. This results in flies being released in clumps, which reduces quality (24). Similarly, Hernandez et al.

(2010) reported that a lower density of *Anastrepha ludens* (Loew) and *Anastrepha obliqua* (Macquart) flies in the release boxes corresponded to an increase in longevity and flight ability post-release (25). Furthermore, a higher density of immobilised fruit flies has been shown to reduce mating competitiveness of the male flies after release (26-27). Our results with *Anopheles* mosquitoes suggest that the effect of compaction upon post-immobilisation survival is minor. This would allow for a greater density of adults within each release cassette. The process of chilling itself is thought to increase condensation, which results in the insects getting stuck to the walls of the release cassette unless sufficient ventilation is available. The transport of *An. arabiensis* will require a ventilation system, as this species has been observed to ‘leg lock’ when held in bulk in an immobile state (NJ Culbert, pers. obs.). The specific impact of condensation remains to be investigated.

Anoxia or oxygen deprivation interferes with the physiological mechanisms involved in insect thermal tolerance (28). Insects are known to adjust the rate at which they produce metabolic heat in response to environmental stress such as anoxia or exposure to temperatures outside their normal range (29). Immobilising adult mosquitoes, whilst subjecting them to a potentially anoxic environment during transport, therefore likely exerts stress upon them. This may in turn influence the amount of metabolic heat they produce and thus the transport temperature itself. Our preliminary study suggested that adult mosquitoes do not emit significant metabolic heat when chilled and held in an immobile state. This finding should be confirmed using a metabolic chamber as previously described by Huestis et al. (2011) to calculate the metabolic rate of *An. arabiensis* (30).

The Tropical Medicine Research Institute (TMRI) in Khartoum, Sudan, with the technical and financial support of the FAO/IAEA, commenced a study in 2004 to assess the feasibility of integrating SIT as part of an AW-IPM strategy. It aimed to manage a population of the malaria vector *An. arabiensis* at carefully selected breeding sites along the River Nile, from Dongola to Merowe (31). Preliminary data confirmed the ability of irradiated and released male *An. arabiensis* to participate in swarms and showed an estimated population density of 12-47 wild males per km<sup>2</sup> (32). These initial data are a useful starting point to estimate sterile male release densities per surface area and required packing densities in the release cassettes. For example, to release 1 000 males per dose, the volume of the release cassette would need to be 7.5 ml, held immobile at between 4 and 10 °C for no longer than 24 h. This would allow males to be produced in Khartoum and transported as adults to Merowe for release, either by air, which will take 1 h, or by road taking approximately 6 h.

We found no effect of immobilising male mosquitoes between 4 and 10 °C, for up to 24 h, on survival up to 14 days post-chilling. Further studies are needed to assess the effects of combining these chosen treatments with irradiation. More complex parameters, such as male competitiveness following immobilisation and/or compaction, also warrant investigation. Sterile males first need to survive the journey from the rearing or release centre to the field, and then they must compete with their wild male counterparts in order to achieve population suppression. It will also be important to replicate these studies with each species of mosquito that is considered for aerial release. The results of this study will assist in the future development and design of aerial release systems for mosquitoes.

In conclusion, the experimental work outlined in this paper has revealed a suitable transportation temperature range and time frame upon which immobilised adult male *An. arabiensis* can be maintained without any immediate impact on survival or subsequent longevity. Immobile adults appear to tolerate a moderate level of compaction without a significant effect on longevity. This is important for SIT. These results will help develop parameters for successful transportation of immobile adult mosquitoes prior to an aerial release. To transport *An. arabiensis* adults for release without impacting survival or longevity we recommend chilling at 4-10 °C for no more than 24 h, providing ventilation, especially if compacting insects in release cassettes. As well as sensitivity to low temperature and impact of compaction, relative humidity and need for ventilation must be considered when designing the release device.

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## Chapter 3

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# Investigating the Impact of Chilling Temperature on Male *Aedes aegypti* and *Aedes albopictus* Survival

The results presented in this chapter have been published as the manuscript:

Culbert NJ, Gilles JRL, Bouyer J (2019) Investigating the impact of chilling temperature on male *Aedes aegypti* and *Aedes albopictus* survival. PLoS ONE 14(8): e0221822.

## Abstract

In genetic control programmes, including the sterile insect technique (SIT), it is crucial to release insects of the highest quality with maximum survival. It is likely that male mosquitoes will follow the trend of other insects in SIT programmes and be stored, transported and eventually released under chilled conditions. The aim of our study was to investigate the impact of different chilling temperatures on male *Aedes aegypti* and *Ae. albopictus* survival by exposing them to a range of temperatures for different durations. *Ae. aegypti* were found to be less sensitive to the impact of chilling, with only 6°C causing a marginal decrease in survival in comparison to non-chilled controls. Conversely, *Ae. albopictus* displayed a significantly reduced survival at all chilling temperatures even when exposed for a short time. In both species, longer exposure to low temperatures reduced survival. Our results uncovered that *Ae. albopictus* are more sensitive to chilling, regardless of the temperature, when compared to *Ae. aegypti*. Such results indicate differences in thermal tolerances between species and the necessity of conducting experiments on a species by species basis when determining temperature limits for any insect destined for release as part of a genetic control programme.

## Introduction

The global burden of vector-borne diseases is increasing with mosquito borne diseases responsible for more than 700,000 deaths each year (179)). With traditional vector control methods such as insecticides becoming less effective due to a build up of resistance in wild populations, alternative vector control tools are needed urgently (2]). Genetic control techniques such as the sterile insect technique (SIT) has seen reignited interest in recent years as a potential method to control mosquitoes as part of an area-wide integrated pest management (AW-IPM) strategy, having been used successfully against various plant and animal pests for over six decades (3).

Significant progress has been made in the last decade towards taking mosquito SIT to the operational level (4). Mass rearing methodology has been developed and standardized, including larval rearing equipment (5-6), larval diet (7) and more recently a rapid quality control device based on flight ability (8). However, distinct gaps still remain in the literature, especially regarding the post-pupal irradiation stages or the handling, transport and release of sterile male mosquitoes. All of which are essential components of A SIT programme and, if not addressed correctly, could impact sterile male quality and survival (9). Historically, the aerial release of sterile insects was carried out by deploying biodegradable release cartons filled with known quantities of insects which minimised the impact of handling and thus preserved quality (10). Today, sterile insects such as fruit flies (*Ceratitis capitata*) and tsetse flies (*Glossina palpalis gambiensis*) are released via a new continuous release system, releasing the chilled adults at a defined rate per surface area (11). During the transportation phase, prior to an aerial release, fruit flies and tsetse flies remain immobilised within a temperature range of 8 to 10 °C (9, 12),

To ensure the success of any release campaign, it is essential to release insects with a maximal quality. As mosquitoes are small bodied poikilotherms, it comes as no surprise that temperature is consistently noted as a key element impacting survival (13). The phenomenon of inducing immobilisation at a species-specific temperature is referred to as the critical thermal minimum ( $CT_{min}$ ) or knockdown temperature where the righting response is lost and the insect is unable to stand up or cling to a surface. This precedes a stage known as a chill-coma where a reversible cessation of movement occurs (14). It has been shown that exposing insects to temperatures out of their normal range can cause stress and in turn reduces their quality and competitiveness (15). Thus, it is fundamental to assess the effects of immobilizing male mosquitoes across an array of temperatures to define a suitable range within which any potential negative impact is mitigated. It is documented within the literature that activity will cease in *Aedes aegypti* at temperatures below 10 °C (16-17).

Previous studies conducted within our laboratory have already suggested a suitable temperature range in which to transport *Anopheles arabiensis* (18). We endeavoured to build on this research and define an optimal temperature range for storing and transporting immobile male *Ae. aegypti* and *Ae. albopictus*. We aimed to determine the temperature thresholds above which, immobilisation would not occur and below which irreversible damage arises and thus impedes subsequent survival and quality. We exposed male *Ae. aegypti* and *Ae. albopictus* to a range of cold storage temperatures for various time periods and monitored their survival post-chilling.

## **Materials and methods**

### **Mosquito colony sources and mass rearing procedures**

The strains of *Ae. aegypti* and *Ae. albopictus* used in the present study were sourced from Juazeiro, Brazil and provided by Biofabrica Moscamed and Rimini, Italy and provided by the centro agricoltura ambiente (CAA) in Crevalcore, Italy respectively. Both *Ae. albopictus* and *Ae. aegypti* have been subsequently reared in the Food and Agricultural Organisation/ International Atomic Energy Agency (FAO/IAEA) Insect Pest Control Laboratory (IPCL) in Seibersdorf, Austria since 2010 and 2012 respectively without further colony regeneration.

Adults were maintained under controlled temperature, relative humidity and light regimes ( $27 \pm 1^\circ\text{C}$ ,  $70 \pm 10\%$  RH, 12:12 h light:dark (L:D) photoperiod, with two one-hour twilight periods simulating dawn and dusk as described in (19). Eggs used for all experiments were generated and hatched based upon standardised guidelines developed at the IPCL (20). Larvae were mass-reared in a climate-controlled room with temperature and RH held constant at  $30 \pm 1^\circ\text{C}$ ,  $70 \pm 10\%$  RH, respectively. Larvae were reared in mass rearing trays with approximately 18,000 first instar ( $L_1$ ) per tray in 5 l of deionized water. 7.5% IAEA diet was administered daily (50 ml on day 1, 100 ml on day 2, 150 ml on day 3, 200 ml on day 4 and 50 ml from day 5 onwards) (21). Pupae were sexed mechanically using a Fay-Morlan (22) glass plate separator subsequently redesigned by Focks (John W. Hock Co., Gainesville, FL, USA (23)), prior to further examination under a stereomicroscope for further accuracy. Adults were maintained in small plastic Bugdorm cages (Bugdorm, Taipei, Taiwan; 15 x 15 x 15 cm) in batches of 30 males per cage with continuous access to a 10% sucrose solution. The experiment commenced on day 2 post-emergence.

### **Determining the experimental temperature range**

Short preliminary experiments were conducted to determine experimental temperatures. Bugdorms of 30 adult *Ae. aegypti* and *Ae. albopictus* were placed inside a climate chamber (Sanyo MLR-351H, Osaka, Japan) with a Hero4 GoPro camera for 20 min to test each temperature across the range of 2 - 14 °C. As immobilisation did not occur above 12 °C, a narrower range of 2 – 10 °C was selected for further investigation. To monitor the temperature inside the climate chambers, Data loggers (Onset Hobo data loggers, Bourne, MA, USA) were placed inside.

### **The impact of chilling temperature on survival**

Bugdorms containing 30 adult male *Ae. aegypti* (3 replicates for each temperature and duration) were maintained inside a climate chamber programmed to 4, 6, 8 or 10 °C, RH 50%, for 1, 4, 8 or 24 h; control adults were maintained at  $25 \pm 1$  °C. Immediately after cold exposure, experimental males were returned to  $25 \pm 1$  °C. The experiment was repeated for *Ae. albopictus* but at 2, 4, 6 and 10 °C. A slightly lower temperature (2 °C) was selected for *Ae. albopictus* as we expected that they would be more tolerant to cold temperatures. Immediate mortality was assessed following immobilisation and survival monitored for a further 14 days, with dead adults removed daily. Survival was not monitored beyond 14 days as survival post-release is not likely to exceed this timeframe.

### **Statistical Analysis**

Binomial linear mixed effect models were used to analyze the impact of the various temperatures and durations on survival rates on day 15 post-exposure (response variables). Temperature and duration were then used as fixed effects and the repetitions as random effects. The temperature of 25°C and the duration of one hour

were set as reference levels (control) in all models and other treatments were compared to these values. The significance of fixed effects was tested using the likelihood ratio test (24-25) and are reported in Tables 1 and 2.

## Results

### The effect of temperature and duration on male *Aedes* survival

In *Ae. aegypti*, temperature did not significantly reduce survival 15 days after immobilisation when exposed to all temperatures for one hour (Fig 1; Table 1,  $p > 0.05$ ) except at 6 °C where a marginally significant effect was observed ( $p = 0.05$ ). With an increase in duration of chilling however, there was a subsequent decrease in survival at all temperatures ( $p < 10^{-3}$ ).

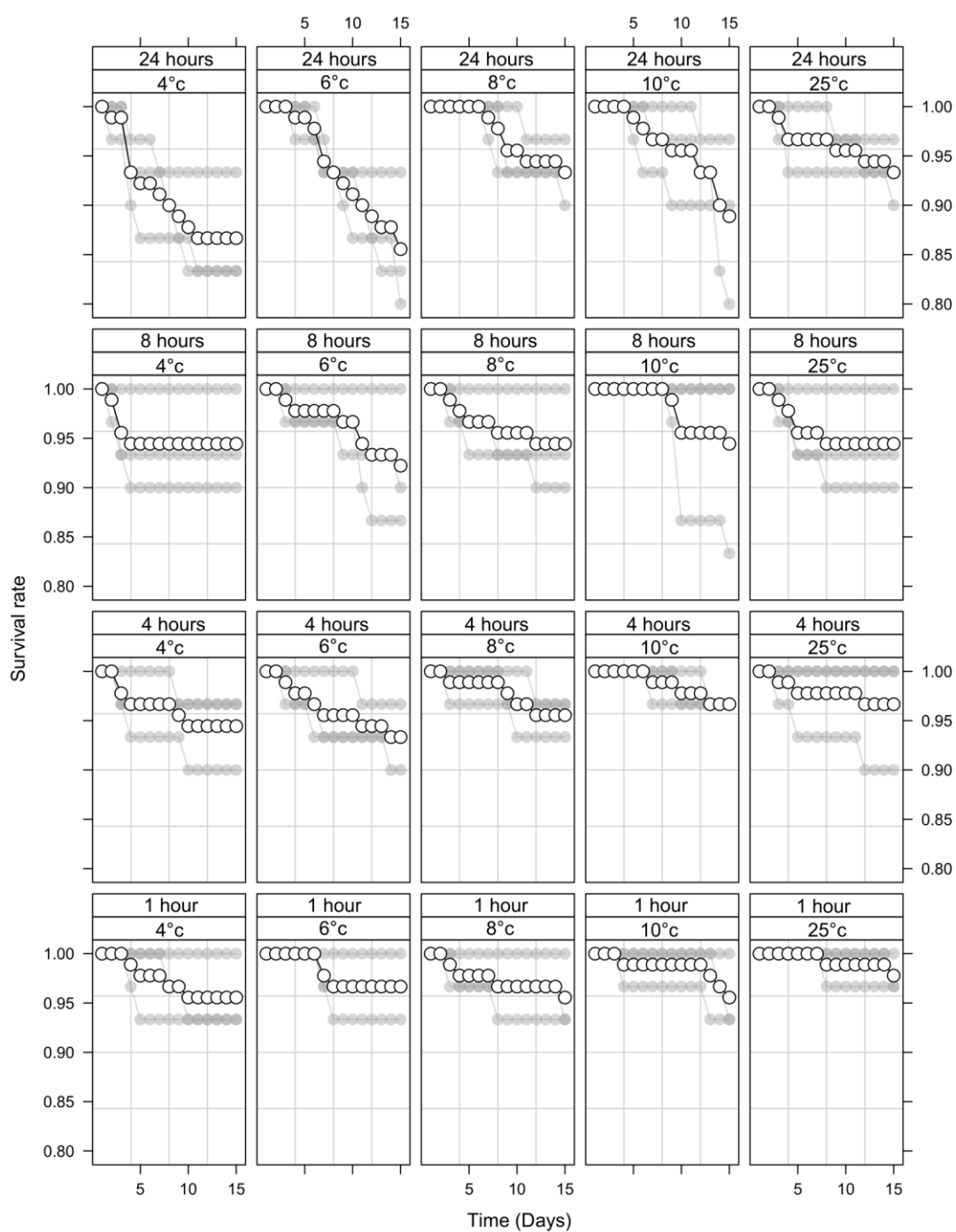
**Table 1:**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	3.55420	0.28728	12.372	2e-16
4 °C	-0.52051	0.32859	-1.584	0.1132
6 °C	-0.64037	0.32253	-1.986	0.0471
8 °C	-0.18227	0.3494	-0.522	0.6019
10 °C	-0.33936	0.33901	-1.001	0.3168
Duration	-0.04433	0.01007	-4.403	1.07e-05

Fixed-effects coefficients of a mixed-effect binomial model on the impact of temperature and duration on the survival of male *Aedes aegypti* on day 15 post-exposure.



**Figure 1:**



Mean ( $\pm$  standard error, SE) % survival of adult male *Aedes aegypti* 15 days following immobilisation at 4, 6, 8 and 10 °C for 1, 4, 8 or 24 h. Mean values are represented by white circles with a black outline. Each series of grey dots represents a repetition (one cage. of 30 adult males).

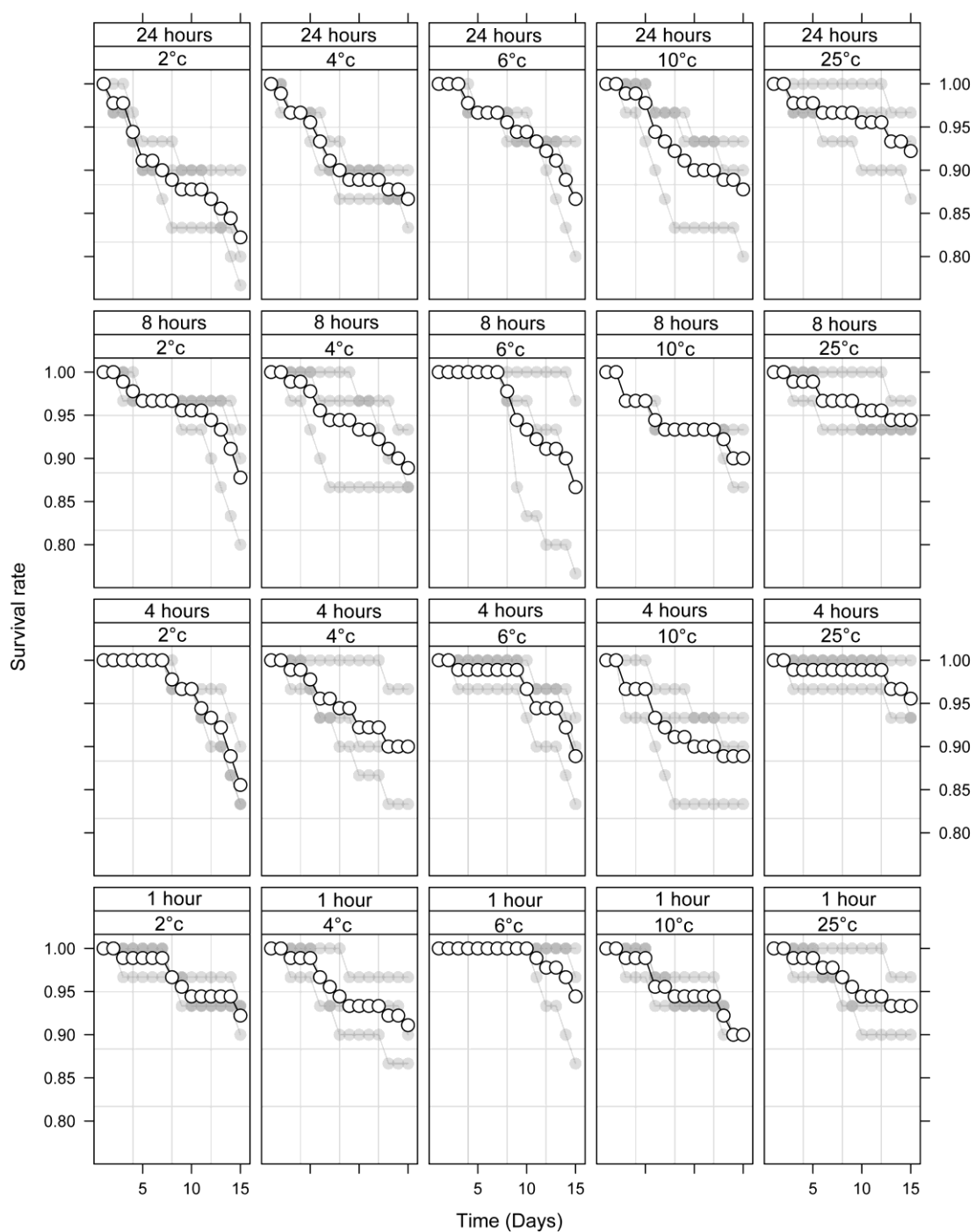
In *Ae. albopictus*, there was a significant decrease in survival 15 days after immobilisation at all temperatures to which they were exposed (2, 4, 6 and 10 °C, Fig 2; Table 2,  $p < 0.03$ ), with duration again further reducing survival with each increase in duration ( $p = 0.02$ ). We therefore suggest that *Ae. aegypti* could be stored immobile between 7 and 10 °C without negatively impacting their survival, at least for short durations, yet further testing is warranted for extended periods of immobilisation. We would be keen to repeat our experiments with *Ae. albopictus*, especially at the higher end of the immobilisation threshold (6 – 10 °C) to see if our results hold true and if there are differences between strains.

**Table 2:**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	2.924461	0.237463	12.315	2e-16
2 °C	-0.838245	0.270320	-3.101	0.00193
4 °C	-0.625690	0.278129	-2.250	0.02447
6 °C	-0.625690	0.278129	-2.250	0.02447
10 °C	-0.625690	0.278129	-2.250	0.02447
Duration	-0.019357	0.008331	-2.323	0.02015

Fixed-effects coefficients of a mixed-effect binomial model on the impact of temperature and duration on the survival of male *Aedes albopictus* on day 15 post-exposure.

**Figure 2:**



Mean ( $\pm$  standard error, SE) % survival of adult male *Aedes albopictus* 15 days following immobilisation at 2, 6, 8 and 10 °C for 1, 4, 8 or 24 h. Mean values are represented by white circles with a black outline. Each series of grey dots represents a repetition (one cage. of 30 adult males).

## Discussion

The results of our study show stark differences in survival post-chilling between *Ae. aegypti* and *Ae. albopictus*. Our previous study highlighted that male *An. arabiensis* could be held at 2 °C for up to 8 hours or between 4 and 10 °C for up to 24 hours without significantly impacting subsequent survival 14 days after immobilisation, except a marginally significant reduction at 6 °C which might be due to experimental uncertainties. This result is consistent with what was found in an earlier study where male *Ae. aegypti* were exposed to 0, 4, 8 and 10 °C for a period of 2 hours with post-chilling survival monitored for a period of 15 days [8]. Only exposure to 0 °C was found to significantly decrease survival whilst 4, 8 and 10 °C did not as per our current study. The susceptibility of *Ae. albopictus* to all chilling temperatures is in stark contrast.

It is postulated that the expanding range of *Ae. albopictus* is attributed to its ability to survive at low temperatures whilst the more limited spread of *Ae. aegypti* is due to its inability to withstand colder conditions. This has been shown in larval survivorship in the 2 species exposed to low temperatures with *Ae. albopictus* displaying a higher survival than *Ae. aegypti* [26]. However, a review of the literature by Brady et al, also concluded that *Ae. aegypti* have a greater tolerance to lower temperatures when compared to *Ae. albopictus*, again in direct contrast to their observed geographic distribution [27]. This result may be explained when considering the egg stages of each species. The eggs of *Ae. albopictus* are capable of undergoing diapause and thus allowing the species to overwinter [28]. On the other hand, *Ae. aegypti* eggs show far less adaptation to survive beyond their normal thermal limits [29]. Thus, there may be a stronger selection pressure in adults to withstand a wider temperature range to resist diurnal and inter-seasonal variations in temperature [27]. A further, more recent review by Schmidt et al, investigating the relationship between humidity and adult survival

with temperature as a modifying effect, concluded that the lowest mortality risk for *Ae. albopictus* and *Ae. aegypti* was at 21.5 and 27.5 °C respectively [30]. Despite the optimum temperature being lower in *Ae. albopictus*, it was noted that *Ae. aegypti* had a survival advantage under most of the tested conditions. Therefore, accounting for humidity may offer an explanation for the differing results reported in the reviews by Schmidt and Brady. Humidity also remained constant during our studies and thus, it may be that *Ae. albopictus* may survive better when chilled at lower temperatures if the level of humidity is adjusted. Further investigation is necessary. It is also worth noting that both reviews were based upon adult female survival whilst our studies only involved males.

Insects are well known to display a high level of variability when it comes to cold tolerance, both between and within species. This phenomenon has been reported in *Aphidiinae* [31] and *Trichogramma* species [32] with even inter-population variability in cold tolerance reported in the latter [33]. Cold tolerance can vary within one population due to epigenetic changes. This is especially true of *Ae. albopictus*, where a local short-term mechanism of the heritable trait of cold hardiness has been suggested for its successful spread into cooler climates [34]. It may be that the strain of *Ae. albopictus* we used for our study, which originated from Rimini, Italy, are less cold tolerant than other strains. The results from our studies concerning both *Ae. aegypti* and *Ae. albopictus* highlighted how determining conditions for one species does not mean it can be inferred for another. *Aedes* species appeared to be less tolerant to low temperatures, even for short durations than *An. arabiensis*, with *Ae. albopictus* displaying a greater sensitivity than *Ae. aegypti*. We would advise to maintain an immobilisation temperature of between 7 and 10 °C when storing and transporting *Ae.*

*aegypti* for short periods of time. With the recent development of our novel quality control tool [8], it may be of value to repeat these studies and follow up with flight ability tests to ascertain if chilling temperature is having a similar effect on their quality, especially in *Ae. albopictus*. Such a high natural variability between species therefore means that individual studies are necessary to determine species-specific parameters for storage and transportation for any insect and, most likely, any strain when considering them for release as part of a genetic control programme.

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## Chapter 4

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# A Rapid Quality Control Test to Foster the Development of Genetic Control in Mosquitoes

The results presented in this chapter have been published as the manuscript:

Culbert N, Balestrino F, Dor A, Herranz GS, Yamada H, Wallner T, et al. A rapid quality control test to foster the development of genetic control in mosquitoes. *Scientific Reports*. 2018;8:16179.

## **Abstract**

Vector-borne diseases are responsible for more than one million deaths per year. Alternative methods of mosquito control to insecticides such as genetic control techniques are thus urgently needed. In genetic techniques involving the release of sterile insects, it is critical to release insects of high quality. Sterile males must be able to disperse, survive and compete with wild males in order to inseminate wild females. There is currently no standardized, fast-processing method to assess mosquito male quality. Since male competitiveness is linked to their ability to fly, we developed a flight test device that aimed to measure the quality of sterile male mosquitoes via their capacity to escape a series of flight tubes within two hours and compared it to two other reference methods (survival rate and mating propensity). This comparison was achieved in three different stress treatment settings usually encountered when applying the sterile insect technique, i.e. irradiation, chilling and compaction. In all treatments, survival and insemination rates could be predicted by the results of a flight test, with over 80% of the inertia predicted. This novel tool could become a standardised quality control method to evaluate cumulative stress throughout the processes related to genetic control of mosquitoes.

## Introduction

Vector-borne diseases account for 17% of infectious diseases leading to more than one million deaths each year(181). The toxicity and ecotoxicity of insecticides together with the spread of resistances to pyrethroids urge the development of alternative mosquito control methods, particularly against *Aedes* vectors. In their global vector control response 2017-2030, the World Health Organization (WHO) indicates the urgent need for alternatives (2). Many new mosquito control methods are thus being tested (1), among which genetic control shows promises (3).

The sterile insect technique (SIT) is a birth control method based on repeatedly releasing large numbers of sterile male insects to reduce the reproduction in a target population of the same species (4). For over six decades, the SIT has been implemented globally through area-wide integrated pest management programs (AW-IPM) to suppress, contain, prevent or even eliminate insect pests of agricultural and medical/ veterinary importance, such as fruit flies, screwworms and tsetse flies (5-7). Despite promising results from initial pilot studies (8), research on mosquito SIT dwindled. However, with current control methods falling below par, together with a lack of effective vaccines, an interest in SIT as a new tool within mosquito AW-IPM programs has been reignited (1).

Reaching the operational level in any SIT program is no easy feat. Establishing mass rearing techniques, standardising irradiation methods, developing a stable sexing system and masterminding release technology are, to name but a few, all essential criteria which must be fully understood in order to achieve a successful program. Furthermore, in AW-IPM approaches that contain an SIT component, the quality of

the sterile insects remains one of the fundamental criteria for a successful program (9). Sterile male insects have one goal and that is to mate with wild females and induce sterility within the target population. Poor quality males may have damaged wings, missing limbs or a shortened lifespan and thus will be unable to compete with wild males in the field. Maintaining high quality management of sterile males is crucial to counteract the reduced field performance that arises from the stress-related impacts of biological or operational attributes such as mass rearing, irradiation, handling, transport and release processes (8).

For many years, SIT was seen as a numbers game and if a program exhibited signs of failure, the thought process was simply to release *more* insects to compensate (182). This was due to an absence of a means to evaluate the effectiveness of mass reared sterile insects and interactions with their wild counterparts, with quality control tools only coming into practice later (10). Today, quality control systems are well established for the production and release of various species of sterile insects (11-12). Insect quality must be routinely assessed and if necessary, improved, via a series of bioassays during the production process within a mass rearing facility. Life history parameters such as egg hatch rate, developmental time, pupal size, sex ratio, adult emergence percentage, longevity are regularly measured. Furthermore, the quality of sterile insects post-release must be assured by evaluating flight ability, dispersal capability, sperm transfer, mating propensity and competitiveness (9). There is a distinct lack of quality control methods to evaluate the quality of sterile male mosquitoes. Current systems routinely involve arduous laboratory, semi-field and field tests, such as mark-release-recapture (MRR) studies to ascertain dispersal, longevity

and competitiveness (13-14). Thus, the demand for quick, cost-effective quality control tools is increasing.

Insect flight ability is known to be a direct, reliable marker of insect quality (15-16). Tools such as flight mills (17), already exist for assessing mosquito flight ability but would simply not be practical for routine use in a mass rearing facility or field site. However, for sterile fruit flies, tsetse flies and moths, flight cylinders, normally composed of PVC tubes are used to gauge flight ability, which has been demonstrated to be a good proxy of mating competitiveness (18-19). Flight cylinders are inexpensive, quick and portable, enabling routine quality tests to be carried out both during the production chain and post-release. Recently, new quality control devices have been designed to infer the survival and mating capacity of radio-sterilized *Aedes albopictus* males through the observation of flight capacity of newly emerged adults from individual pupae (20). This test was however time consuming (48H to 72H) and did not allow measuring the impact of various treatments to which adults are subjected from their production to their release. In order to improve the practicality, manoeuvrability and response time of the flight organ devices, a new flight cylinder device capable to test batches of 100 adults directly within a two hour period without introducing them at pupal stage was proposed. We present the results of a series of validation tests during which *Ae. albopictus* and *Ae. aegypti* adult mosquitoes were subject to varying levels of stress treatments which are known to affect mosquito quality, including irradiation, chilling and compaction (9). Flight ability was subsequently measured and compared to the results of mating capacity and survival which were measured as reference tests.

## **Materials and Methods**

### **Mosquito Colony Rearing**

The strains of *Ae. aegypti* and *Ae. albopictus* used in all experiments originated from Juazeiro, Brazil and Rimini, Italy, respectively. They were transferred to the Food and Agricultural Organisation/ International Atomic Energy Agency (FAO/IAEA) Insect Pest Control Laboratory (IPCL) in Seibersdorf, Austria by Biofabrica Moscamed, Brazil and Centro Agricoltura Ambiente “G.Nicoli” (CAA), Italy respectively. They are maintained in climate controlled insectary (temperature  $27 \pm 1^\circ\text{C}$ , relative humidity  $70 \pm 10\%$ , photoperiod 12:12, with two one-hour twilight periods simulating dawn and dusk) as was previously described by (24). For all experiments, larvae were reared in plastic trays (40 x 29 x 8 cm) containing 1 litre of deionized water at a density of approximately 3000 first instar ( $L_1$ ) per tray and were provided with the IAEA-2 diet following the protocol described in (33-34).

### **Irradiation Procedure & Experimental Design**

Pupae were separated from larvae and sexed mechanically (John W. Hock Co., Gainesville, FL) prior to further examination under a stereomicroscope, ensuring pure batches of males and females. Male pupae were irradiated at  $36 \pm 4$  hours in batches of 150 inside a self-contained  $^{60}\text{Co}$  Gamma Cell 220. Dose accuracy was measured with a dosimetry system using Gafchromic MD film. A range of irradiation doses were selected for each species, including the values necessary to induce full sterility and then beyond to severely reduce the quality of the adults. With 0 Gy representing the controls for each species, 30, 90, 110 and 150 Gy and 20, 40, 80 and 100 Gy were chosen for *Ae. aegypti* and *Ae. albopictus* respectively.

Adults were maintained in standard plastic cages (30 x 30 x 30 cm – Bugdorm, Taiwan) with continued access to a 10% sucrose solution until day 3 when experiments were performed. Mosquito maintenance and the age of the adults when all described experiments were performed was chosen to reflect what would occur in a mass rearing facility prior to a release of sterile males. There were two replicates for each stress treatment in addition to two control samples for each experiment performed.

### **Chilling Procedure & Experimental Design**

As with irradiation, a range of chilling temperatures were selected for *Aedes aegypti* that were known to be within a tolerable limit and others were chosen with the aim that they would impact quality following exposure. When aged 3 days, batches of 250 adult males were immobilised and held for two hours at 0, 4, 8 or 10 °C with control males left in insectary conditions ( $27 \pm 1$  °C).

### **Compaction Procedure & Experimental Design**

Batches of 250 adult male *Ae. aegypti* were immobilised at 10°C, a temperature known not to impact their quality, for a period of two hours. During this period, they were subject to various levels of compaction by adding 0, 5, 15, 25 or 50 g weights, corresponding to 0, 0.25, 0.76, 1.27 and 2.55 g/ cm<sup>2</sup> respectively. Cumin seeds were wrapped in mesh and sealed with an elastic band to serve as a substitute for mosquitoes during this experiment. The morphological properties and weights of various substitute particles including rice, poppy, anise, fennel and cumin seeds were analysed previously with cumin seeds found to best match the weight and characteristics of adult mosquitoes, hence their selection for this experiment.

### **Assessing Survival Rate and Mating Propensity as a Measure of Quality**

The survival rate and mating capacity of males under each of the aforementioned stress treatments (irradiation, chilling or compaction) were measured with the aim to link these known quality parameters with their flight ability post stress treatment. The survival rate was quantified by removing and counting dead individuals from both control and experimental cages daily for a period of 15 days. The number of adults remaining for longevity assessment (N) varied slightly between experiments. For the irradiation experiments, N varied from 114 – 197 and 64 – 151 for *Aedes aegypti* and *albopictus* respectively. For the temperature and compaction experiments, N varied from 109 – 149 and 118 – 173 respectively for *Aedes aegypti*. Mating propensity was calculated by measuring the number of virgin females a single control or post stress treatment male could successfully inseminate during a period of 5 days. A single adult male mosquito, from each batch of 250 controls and treatment cages, was transferred to a small cage (30 x 30 x 30 cm) containing 10 virgin females from the same cohort. There were 5 repetitions for all treatments and the control and all adults were allowed continued access to a 10 % sucrose solution. Afterwards, each female was dissected and all 3 spermathecae removed to check for the presence or absence of sperm under a stereomicroscope. Females were scored as inseminated and fully inseminated if at least one and two or more spermatheca contained sperm respectively.

### **Flight Test Device and Experimental Procedure**

A flight test device (FTD), which aims to evaluate the flight ability of an adult mosquito, was created after experimental testing (*SI Methods*). The FTD consists of a series of 40 transparent acrylic plastic (Polymethyl methacrylate - PMAA) flight tubes, surrounded by a larger PMAA tube. The first two series of tubes are housed within a



third PMAA tube of greater size which serves as a containment box after mosquitoes escape the flight tubes (see *SI Methods* for complete dimensions). Mosquitoes are blown into the FTD via a mouth aspirator and are given a period of 2 hours to escape. Afterwards, the number of adults that remain at the base of the flight tubes and those that have escaped are counted. Flight ability is calculated by dividing the number of adults which escaped by the total number which entered the flight tube. An average is then calculated across 2 repetitions.

### **Statistical Analysis**

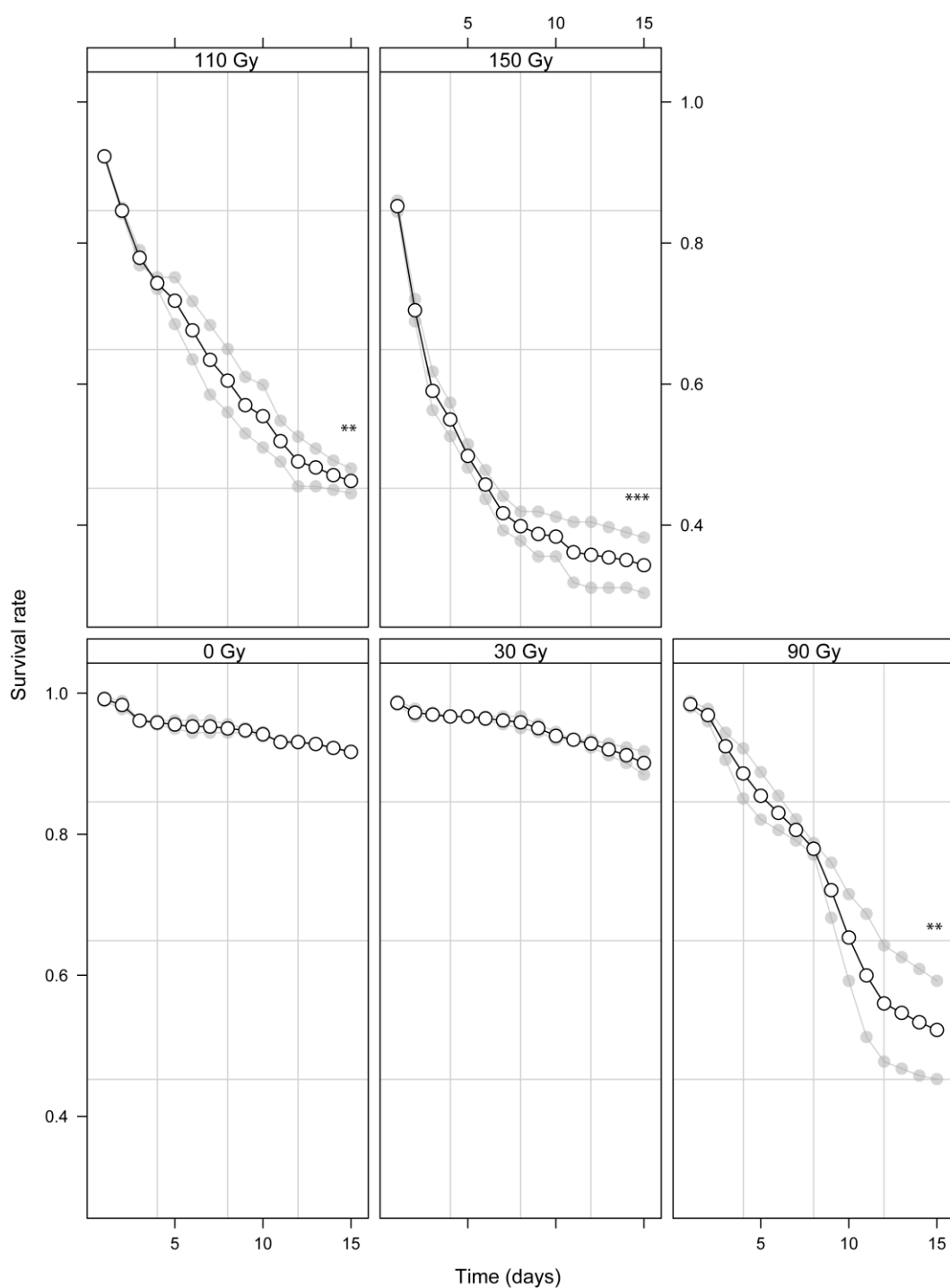
Binomial linear mixed effect models were used to analyze the impact of the various treatments on survival rates at day fifteen, insemination rates, full insemination rates and escape rates from the flight test device (response variables). The treatment regimens for irradiation, chilling and compaction were then used as fixed effects and the repetitions as random effects. The significance of fixed effects was tested using the likelihood ratio test (35-36). We also used binomial linear mixed effect models to analyze how the escape rate could explain the three other quality control parameters (survival rates at day fifteen, insemination rates, full insemination rates). To do so, the quality control parameters were used as response variables and the escape rate as a fixed effect. The  $R^2$  (coefficient of determination) was then used to describe the proportion of variance explained by the model between the observed and predicted values (37-38).

## **Results**

### **Impact of treatments on survival and insemination rates**

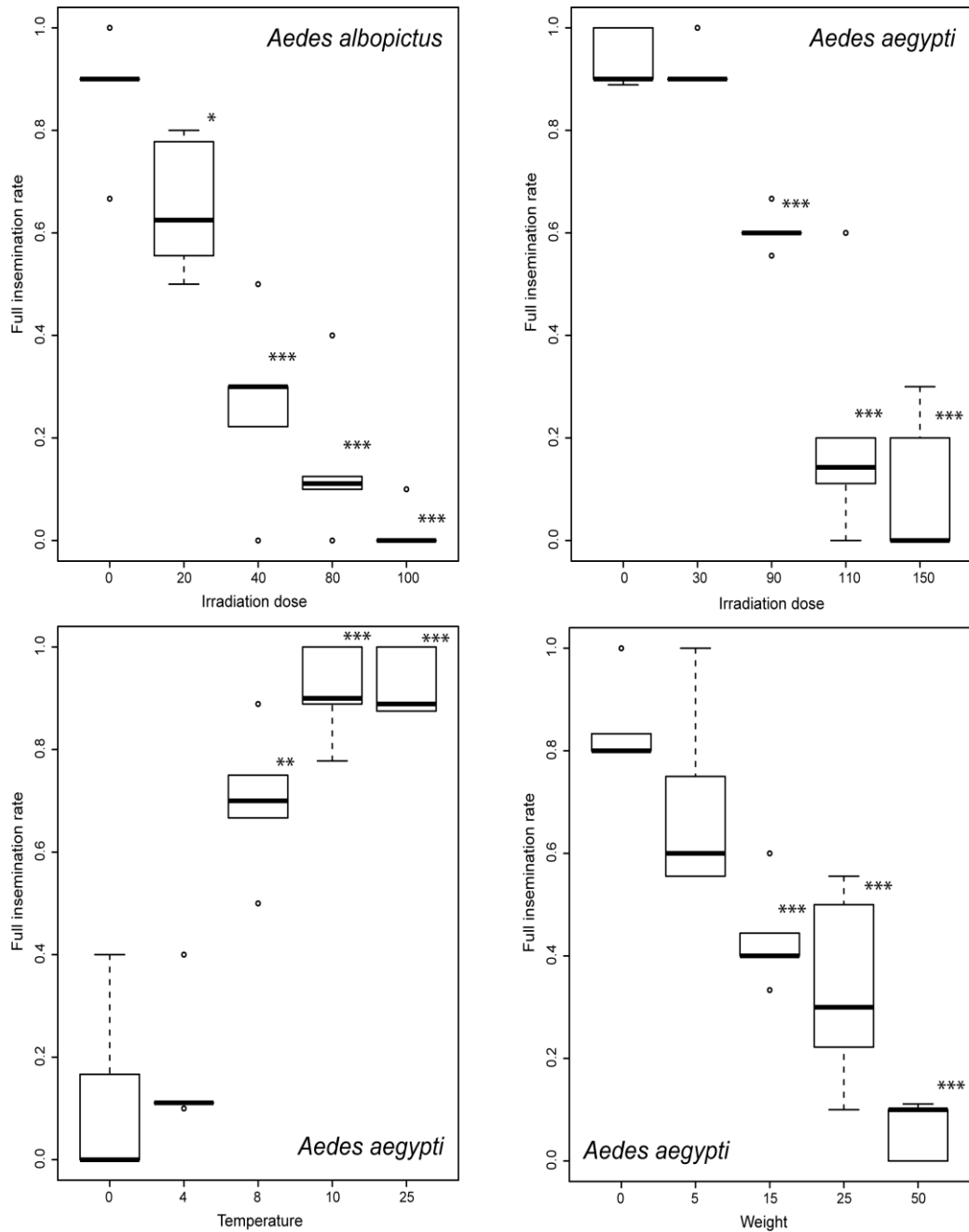
Irradiation reduced survival significantly at a dose equal to or superior than 90 Gy in *Aedes aegypti* (Fig. 1) and 40 Gy in *Ae. albopictus* (Fig. S1). It also reduced the full insemination rate significantly starting from 90 and 20 Gy in *Aedes aegypti* and *Ae. albopictus* respectively (Fig. 2). The insemination rate was less sensitive than the full insemination rate, with a significant decrease in *Ae. albopictus* only, commencing from 40 Gy (Fig. S2). Considering the impact of chilling on male quality in *Ae. aegypti*, the survival rate was significantly reduced only at a temperature of 0 °C (Fig. S3) while the full insemination rate already began declining from exposure to 8 °C. Again, the insemination rate appeared less sensitive than these two aforementioned parameters (Fig. S2). Finally, compaction significantly impacted the survival of *Ae. aegypti* from a weight of 5g (0.25 g/cm<sup>2</sup>) onwards (Fig. S4), illustrating how fragile this insect species is. The full insemination rate was reduced only when the weight exceeded 15 g and the insemination rate was again less sensitive as seen with irradiation and chilling data above (Fig. S2).

**Figure 1:**



Survival rates of male *Aedes aegypti* exposed to various irradiation doses over a period of 15 days. Significant differences between treatment groups (30, 90, 110 and 150 Gy) and the control group (no irradiation) are indicated (\*  $p < 0.005$ , \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ). Individual values of the repeats are indicated in light grey and mean values as a solid line.

**Figure 2:**

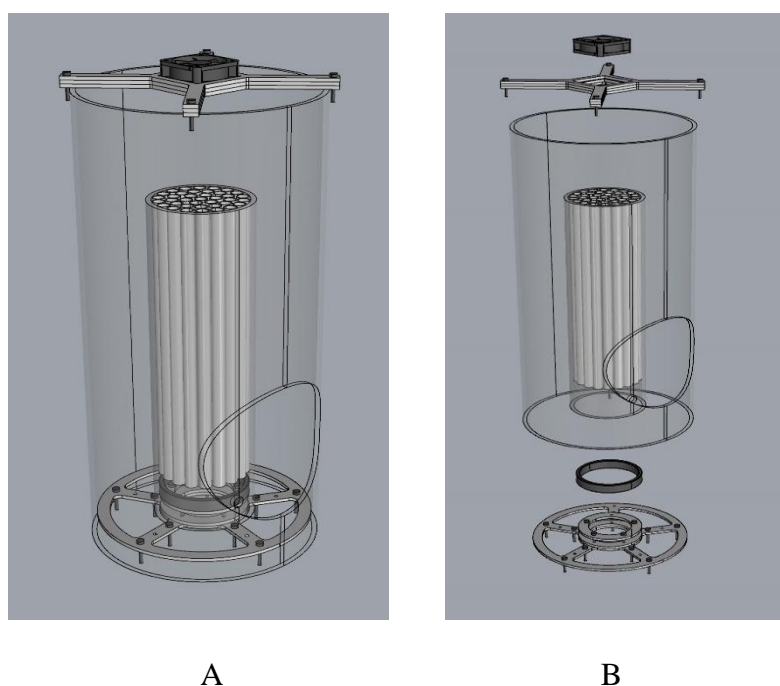


Full insemination rates of male *Aedes* mosquitoes exposed to various treatments. The top panels present the impact of various irradiation doses on *Aedes albopictus* (left) and *Ae. aegypti* (right). The bottom panels present the impact of chilling (left) and compaction (right) on *Ae. aegypti*. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles and dots the minimal and maximal values. Significant differences between treatment groups and the control group are indicated (\*  $p < 0.005$ , \*\*  $p < .01$ ; \*\*\*  $p < 0.001$ ).

### **Flight test device**

Flight ability was measured by aspirating a sample of 100 adult male *Aedes aegypti* or *albopictus* into one of the flight test devices (FTD) via a small 1cm hole at the bottom of the device (see Fig. 3). The mosquitoes are then within a confined space of 1 cm in height and thus their natural instinct is to fly upwards via one of the 40 flight tubes (25cm high, inside diameter of 8mm) and out into the large, containment tube. After filling the FTD with mosquitoes, one small pellet of BG lure (Biogents, Regensburg, Germany) is placed on the top, directly underneath a 12 V fan that is then switched on. The fan speed is 6000 revolutions per minute (rpm) capable of generating an airflow of 11.9 m<sup>3</sup>/ hour. After two hours, the fan is stopped and the experiment is classed as finished. The FTD is then taken to a cold room ( $4 \pm 1^{\circ}\text{C}$ ) and after 5-10 minutes when the mosquitoes are immobile, the number of adults still remaining within the flight tubes or underneath them and those who successfully escaped are counted. The number of escaped males is divided by the total number of males, thus generating an escape rate.

**Figure 3:**



The flight test device (FTD). A complete overview of the FTD in panel A. The placing of each component can be depicted from panel B.

### **Impact of treatments on flight ability**

Flight ability measured as described upon overall appeared as an excellent quality control parameter since it was sensitive to all treatments (Fig. 4). It predicted accurately the different thresholds impacting other parameters (Table. 1), explaining 78 to 92% of the variance of survival rates, 62 to 95% of the variance of insemination rates and 53 to 86% of the variance of full insemination rates. It was interesting to see that the survival rate was more sensitive to the compaction treatment than the full insemination rate whereas the contrary was observed for chilling. Flight ability was in both cases as sensitive as the most sensitive of the two others, with the only exception of irradiation dose in *Ae. albopictus*, which gave a significant reduction of the full insemination rate at 20Gy already whereas it reduced the flight capacity starting from

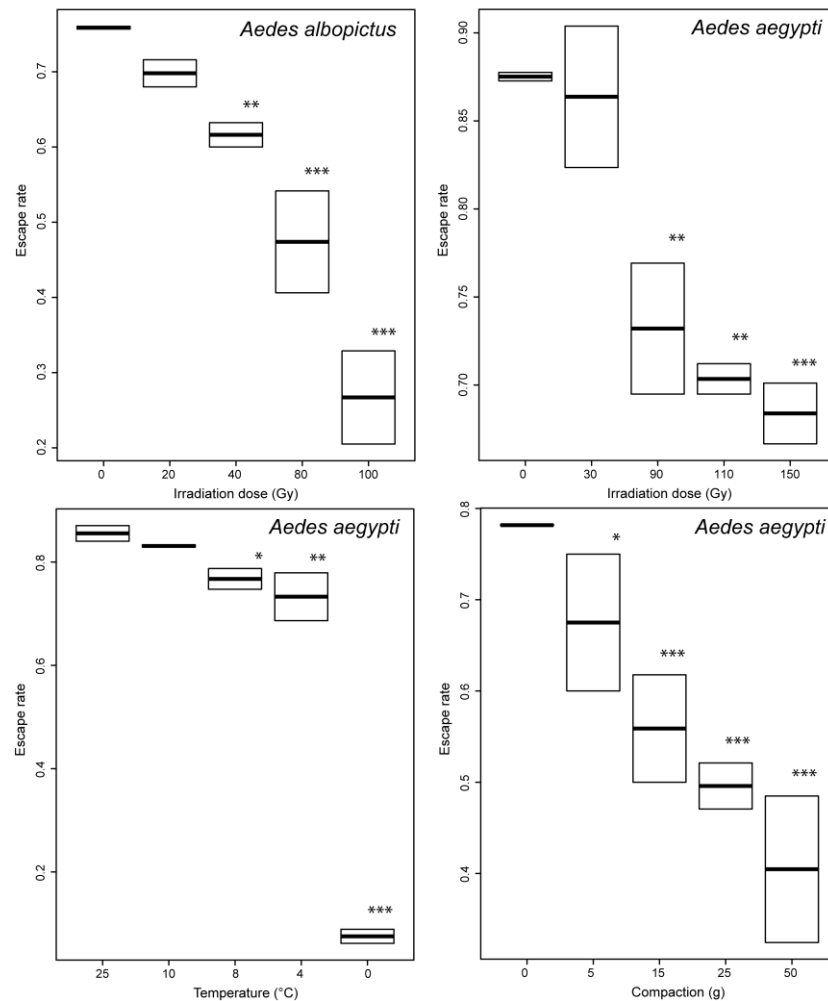
40Gy. For a detailed breakdown of the individual results of each model see SI. Tables 1 – 13.

**Table 1:**

Species	Treatment	First significant impact on escape rate	First significant impact on survival rate at day 15	First significant impact on insemination rate	First significant impact on full insemination rate
<i>Aedes aegypti</i>	Irradiation	90 Gy	90 Gy (0.819)	NA (0.951)	90 Gy (0.840)
	Chilling	8°C	0°C (0.802)	NA (0.616)	8°C (0.532)
	Compaction	5 g	5g (0.776)	NA (0.879)	15g (0.812)
<i>Aedes albopictus</i>	Irradiation	40 Gy	40 Gy (0.918)	40 Gy (0.790)	20 Gy (0.859)

Use of the male escape rates from the flight organ to predict adult male quality parameters. The first values of the different treatments significantly impacting each male quality indicator are presented. The values in brackets correspond to the proportion of explained variance (r-square), used as a model quality indicator, based on a linear mixed-effect model where the response variable (survival, insemination and full insemination rates) is predicted using the escape rate as a fix effect and the repeats as random effects. All p-values of the predictions were below 0.001. Survival was quantified by removing and counting dead individuals from both control and experimental cages daily for a period of 15 days. Mating propensity was calculated by measuring the number of virgin females (n = 10) a single control or post stress treatment male could successfully inseminate during a period of 5 days. Females were scored as inseminated or fully inseminated if one or two or more spermatheca contained sperm respectively.

**Figure 4:**



Escape rates of male *Aedes* mosquitoes exposed to various treatments. The top panels present the impact of various irradiation doses on *Aedes albopictus* (left) and *Ae. aegypti* (right). The bottom panels present the impact of chilling (left) and compaction (right) on *Ae. aegypti*. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles and dots the minimal and maximal values. Significant differences between treatment groups and the control group are indicated (\*  $p < 0.005$ , \*\*  $p < .01$ ; \*\*\*  $p < 0.001$ ).

## Discussion

Inducing sterility in insects is most commonly achieved via ionizing radiation. However, it has been repeatedly reported to impact the subsequent survival and fitness of the insect (8). Thus, the balance between quality and sterility is a delicate one. Administering too low a dose will cause insects to retain high levels of fertility whilst



too high a dose will severely impact the field competitiveness of the insect. High irradiation doses increase the level of somatic damage and thus decrease the quality of the insect which will in turn exhibit reduced mating capacity, flight capacity and longevity. Releasing poor quality insects will decrease the effectiveness of an SIT program, make it more costly and thus require more insects to be released, or the overflooding ratio to be increased (5). It is recommended to select a lower irradiation dose and release a more competitive insect when confronted with this trade off (21). *Ae. albopictus* has been shown to be partially and fully sterile at 35 and 40 Gy respectively whilst still equally as competitive as non-irradiated controls (14, 22-23) thus we chose our irradiation doses based around this knowledge. On the other hand, an absence of irradiation literature regarding *Ae. aegypti* meant that the doses selected were based on personal communications within the IPCL (partially sterilising dose of 90 Gy). Surprisingly, we noted that our standard irradiation doses of 40 and 90 Gy for *Ae. albopictus* and *Ae. aegypti* respectively caused significant decreases in quality in all measured parameters. This is in contrast to previous findings on competitiveness measured in semi-field experiments which might indicate that flight ability is even more sensitive than the latter. It would be of great interest to perform semi-field and field competitiveness tests in parallel to the experiments presented within this paper.

In current SIT programs, insects are routinely exposed to chilling in order to immobilise them to facilitate their handling and eventual field release, such as Mediterranean fruit flies (*Ceratitidis capitata*) which are maintained at 4°C for up to 3 hours prior to an aerial release (19). In contrast to other species of sterile insects, there is a distinct gap in the literature regarding the handling, transport and release of sterile male mosquitoes. Based upon a recent publication (24), and following preliminary

trials within our laboratory with *Aedes aegypti*, we were able to determine a range of immobilisation temperatures for our chilling stress treatment. We predicted that when male *aegypti* were chilled at 8 and 10 °C, they would be of equal quality to controls in contrast to those exposed to 4 and 0°C. Interestingly, our results indicated that only exposure to the lowest chilling temperature, 0 °C, significantly decreased their survival 15 days after exposure, a similar result to what was found in *Anopheles arabiensis* which only exhibited a significantly reduced survival when exposed to 2 °C which was also the lowest chilling temperature within the study (24). However a significant decrease in flight ability was noted after chilling at 8 °C. This is similar to what has been noted in recently emerged tsetse flies after the shipping of chilled pupae at 8 °C for up to 72 hours (18). This is however in contrast to what has been observed in sterile fruit flies where chilling only has a significant impact on flight ability and mating competitiveness when flies are maintained in crowded conditions prior to being chilled for between 0 and 3 hours (19). These results emphasise how chilling can impact sterile insects differently according to species, the duration of chilling or the conditions prior to chilling i.e. crowding, in addition to highlighting the importance of routine quality control checks via devices such as a flight cylinder. It may be of value to conduct tests within the FTD following chilling at different temperatures for varying lengths of time and perhaps densities to try to disentangle the effects of each parameter with regard to male quality and to ascertain if a synergetic effect arises from independent parameters.

Unlike in SIT programs involving tsetse or fruit flies, mosquitoes will be transported to release sites in their adult phase as opposed to pupal. Dealing with the fragility of such an insect poses unique questions. One grey area has been the maximum capacity of adult mosquitoes that can be stored and how tolerant they are to compaction. We

suspected that immobile males would become damaged if the load above them was too high. Our results confirmed that even a weight of 5 g ( $0.25\text{g}/\text{cm}^2$ ), was enough to significantly decrease longevity, which will be of great value when designing transportation boxes or cassettes for adults in addition to the maximum capacity that can be maintained within each box. Overcrowding was found to impose a synergetic effect on fruit flies when flies were held immobile, one which can be reversed by maintaining flies at lower densities. Independently, chilling and crowding did not cause any significant effect upon mating success or flight ability (19).

As mosquito SIT moves closer to an operational level the necessity to accurately determine the quality of sterile males at every point in the production chain and afterwards grows. Our FTD allows a sample of 100 mosquitoes to be sampled in one device, which is significantly higher than current flight mills where a maximum of 16 insects can be sampled at any given time(184). Moreover, we are currently using ten devices simultaneously but due to the low cost, ease of use and few parts necessary to construct the FTD, there is a limitless possibility of how many insects from various cohorts or stages of the mass rearing procedure that could be tested at the same time. Our FTD will thus be a useful and effective tool for monitoring and providing feedback on the quality of sterile male mosquitoes during the production, handling and release phases of a control programme that comprise an SIT component. Our results may also be useful for all strategies based on genetic control that depend on the release of sexually competitive mosquitoes, including *Wolbachia*-infected mosquitoes (26-27), RIDL (28-29) or gene drive (30-31).

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## Supporting Information

### Methods:

**Flight Test Device and Experimental Procedure. *Dimensions.*** The flight test device (FTD) consists of a series of 40 transparent acrylic plastic (Polymethyl methacrylate - PMMA). The height = 25cm, external diameter = 1cm, internal diameter = 0.8cm, wall thickness = 0.1cm. These tubes are encased within a larger PMMA tube (height =

29cm, external diameter = 8cm, internal diameter = 7.2cm, wall thickness = 0.4cm) and gaps between tubes are sealed with silicone gel. There is a 4cm gap at the bottom of this tube with a hole (1 cm diameter) half way up which serves as an entry point for the mosquitoes to be aspirated into the flight tubes. The flight tubes and outer casing are housed within a third, larger PMAA tube (height = 40cm, external diameter = 18cm, internal diameter = 17.2cm, wall thickness = 0.4cm) which contains the mosquitoes after they have successfully exited the flight tubes. This containment tube is closed entirely at the top with a solid mesh. The bottom of the tube has a mesh sleeve where the middle sized PMAA tube containing the flight tubes is inserted and removed. Mosquitoes are aspirated into the base of the PMAA tube to begin a flight ability test via an opening in the middle of the containment tube. This allows them to be directly aspirated into the base to begin the test. This hole is 10 cm in diameter and is surrounded by a mesh sleeve which can be tied closed. The middle-sized tube containing the flight tubes sits on a plastic base to allow sufficient ventilation. Once placed upon the base, the hole where the mosquitoes enter is raised up blocking the entry point and preventing them from escaping. Above the containment tube is a plastic fan holder which holds a 12 V fan in place. Before beginning a flight test, one blue pellet of BG lure is placed directly underneath the fan prior to it being switched on. The fan is connected directly to a power pack. The lure serves as an attractant to encourage the mosquitoes to exit the tubes.

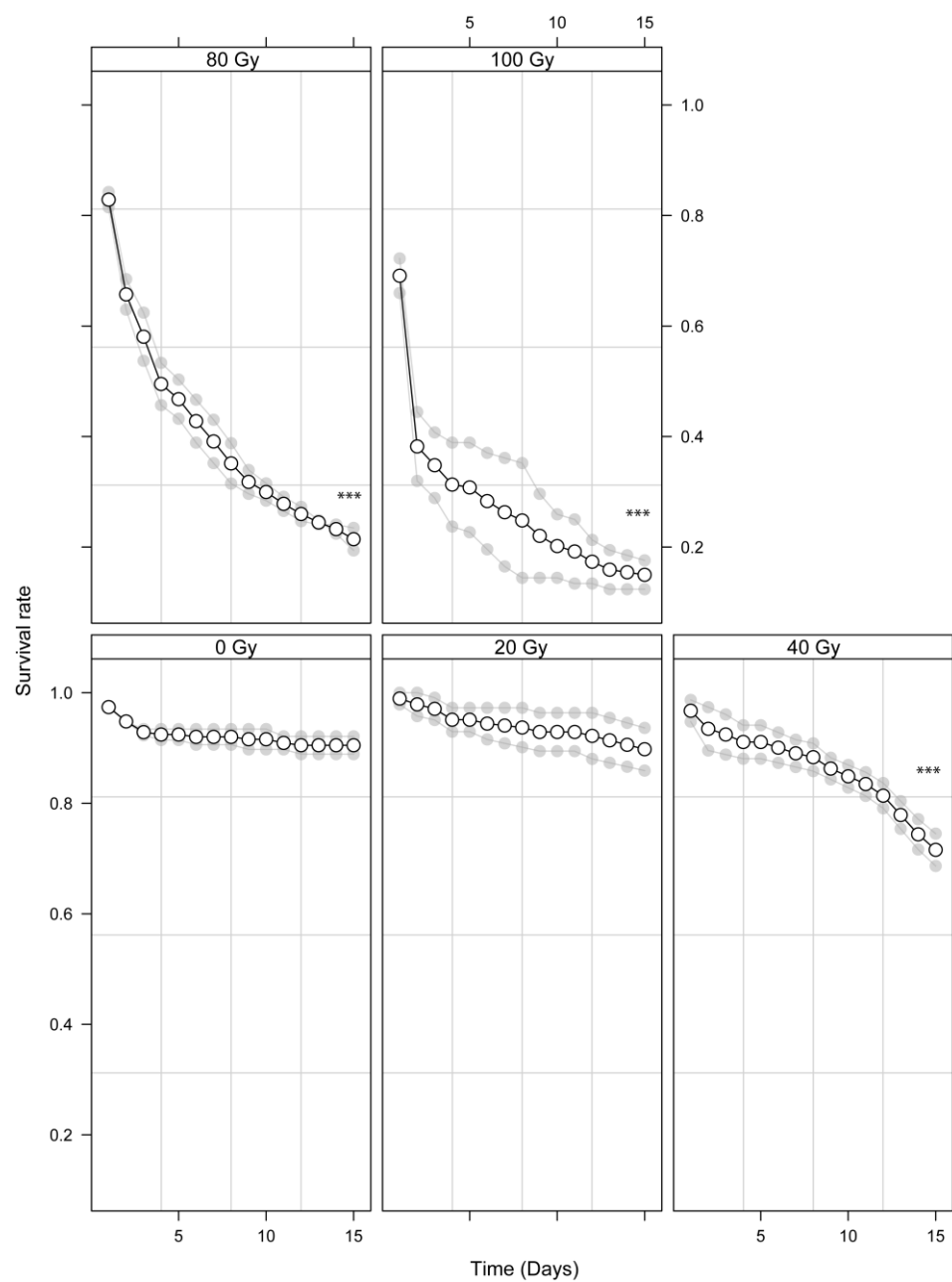
**Flight Test Device and Experimental Procedure. *Prior Laboratory Testing.*** The final design of the FTD was reached after lengthy laboratory testing. Several configurations were tried and tested to develop the optimum dimensions and parameters for the final design. A black cover was used to hide the bottom of the flight

test tubes in one experiment. This idea was rejected as the black coloration served as a resting place and the mosquitoes failed to fly out of the flight tubes. The FTD was tested with and without 5 and 12 V dc fans. Initially tests were carried out with the 5V fan and it did not increase the escape rate of the mosquitoes. However, when a 12V fan was tested, it increased the escape rate, most likely due to the greater air flow and thus was included in the final design. Experiments were carried out with and without BG lure pellets. Initially, the BG lure was not thought to encourage the mosquitoes to exit the flight tubes. This may be due to the experiments being conducted with the 5V fan. When tested with the 12V, the BG lure was found to increase the escape rate. The orientation of the device (horizontal, vertical or 45° angle) was investigated. Horizontal positioning of the FTD enabled the mosquitoes to walk out of the flight tubes instead of flying and thus was rejected. When positioned at a 45° angle, it was again found to be too easy for the mosquitoes to escape the flight tubes, thus a vertical position was chosen. Experiments were conducted to ascertain the optimum length of time allowed within the FTD. Initial results showed a significant increase in escape rate when allowing 18 hours in comparison to 15, 30, 60 and 120 minutes. However, when using the 12V fan and BG lure it was found that 120 minutes was optimum and allowing extra time did not significantly improve the escape rate. Lastly, initial tests were conducted with flight tubes of 40 cm in height (outside diameter 10 mm, inside diameter 8 mm). This was later revised down to 25 cm and found to improve the escape rate significantly.



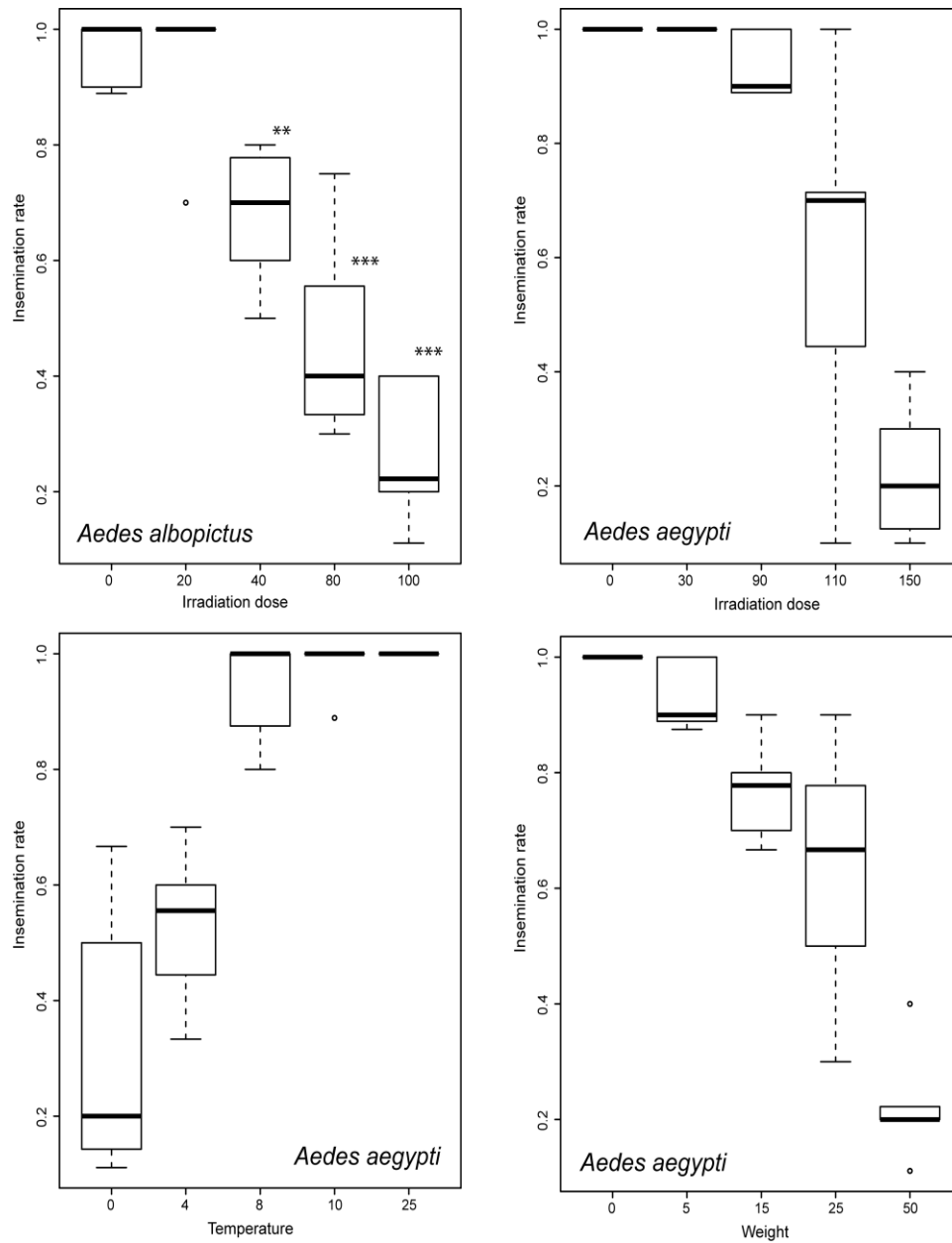
## Results

**Figure S1:**



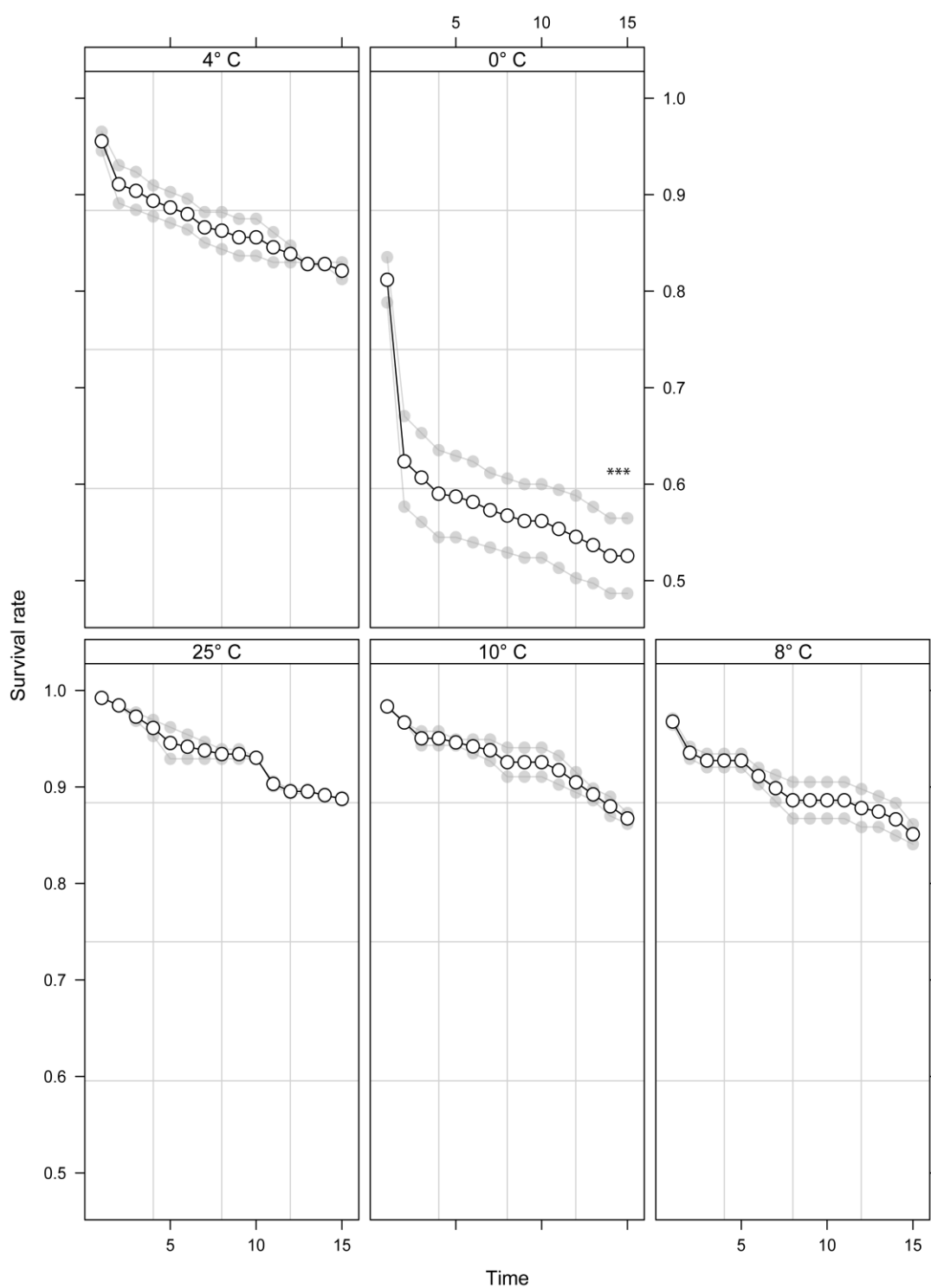
Survival rates of male *Aedes albopictus* exposed to various irradiation doses over a period of 15 days. Significant differences between treatment groups (20, 40, 80 and 100 Gy) and the control group (no irradiation) are indicated (\*  $p < 0.005$ , \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ). Individual values of the repeats are indicated in light grey and mean values as a solid line.

**Figure S2:**



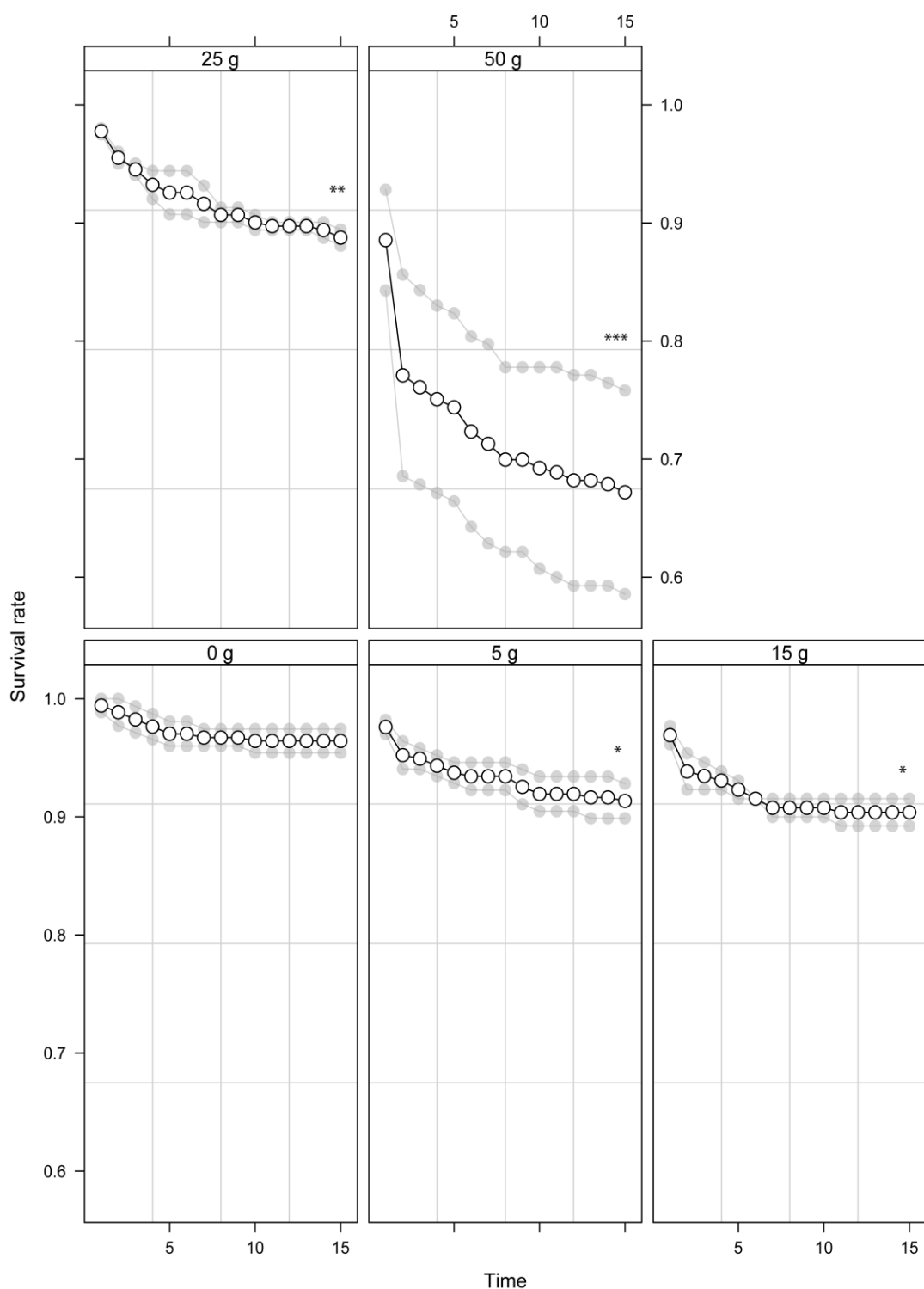
Insemination rates of male *Aedes* mosquitoes exposed to various treatments. The top panels present the impact of various irradiation doses on *Aedes albopictus* (left) and *Ae. aegypti* (right). The bottom panels present the impact of chilling (left) and compaction (right) on *Ae. aegypti*. Boxplots present the median value and the quartiles, horizontal bars the 95% percentiles and dots the minimal and maximal values. Significant differences between treatment groups and the control group are indicated (\*  $p < 0.005$ , \*\*  $p < .01$ ; \*\*\*  $p < 0.001$ ).

**Figure S3:**



Survival rates of male *Aedes aegypti* exposed to various chilling temperatures over a period of 15 days. Significant differences between treatment groups (10, 8, 4 and 0 °C) and the control group (no chilling - 25°C) are indicated (\* p<0.005, \*\* p <.01; \*\*\* p < 0.001).. Individual values of the repeats are indicated in light grey and mean values as a solid line.

**Figure S4:**



Survival rates of male *Aedes aegypti* exposed to various levels of compaction over a period of 15 days. Significant differences between treatment groups (5, 15, 25 and 50 g) and the control group (no compaction – 0 g) are indicated (\*  $p < 0.005$ , \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ). Individual values of the repeats are indicated in light grey and mean values as a solid line.

## Tables:

**Table S1:**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	2.5132	0.2233	11.254	2e-16
30 Gy	-0.1465	0.2747	-0.533	0.591
90 Gy	-2.3992	0.2256	-10.635	2e-16
110 Gy	-2.5056	0.2273	-11.023	2e-16
150 Gy	-2.9113	0.2414	-12.060	2e-16

Fixed-effects coefficients of a mixed-effect binomial model of the impact of irradiation dose on survival in *Aedes aegypti* (10 observations, 2 repeats, 6 degrees of freedom).

**Table S2:**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	2.5200	0.2778	9.071	2e-16
20 Gy	-0.2820	0.3511	-0.803	0.422
40 Gy	-1.4688	0.3097	-4.742	2.11e-06
80 Gy	-3.5748	0.3105	-11.511	2e-16
100 Gy	-3.7955	0.3442	-11.029	2e-16

Fixed-effects coefficients of a mixed-effect binomial model of the impact of irradiation dose on survival in *Aedes albopictus* (10 observations, 2 repeats, 6 degrees of freedom).

**Table S3:**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	2.7300	0.5959	4.582	4.62e-06
30 Gy	-0.2877	0.7917	-0.363	0.716331
90 Gy	-2.3072	0.6650	-3.470	0.000521
110 Gy	-4.0110	0.6949	-5.772	7.82e-09
150 Gy	-4.8818	0.7605	-6.419	1.37e-10

Fixed-effects coefficients of a mixed-effect binomial model of the impact of irradiation dose on full insemination rate in *Aedes aegypti* (25 observations, 5 repeats, 6 degrees of freedom).

**Table S4:**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	1.9700	0.4587	4.294	1.75e-05
20 Gy	-1.3346	0.5393	-2.475	0.0133
40 Gy	-3.0097	0.5526	-5.446	5.14e-08
80 Gy	-3.7203	0.6107	-6.092	1.11e-09
100 Gy	-5.8583	1.1080	-5.287	1.24e-07

Fixed-effects coefficients of a mixed-effect binomial model of the impact of irradiation dose on full insemination rate in *Aedes albopictus* (25 observations, 5 repeats, 6 degrees of freedom).

**Table S5:**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	3.1355	0.7223	4.341	1.42e-05
20 Gy	-0.4729	0.9372	-0.505	0.6138
40 Gy	-2.4116	0.7839	-3.076	0.0021
80 Gy	-3.3098	0.7806	-4.240	2.23e-05
100 Gy	-4.1259	0.7920	-5.210	1.89e-07

Fixed-effects coefficients of a mixed-effect binomial model of the impact of irradiation dose on insemination rate in *Aedes albopictus* (25 observations, 5 repeats, 6 degrees of freedom).

**Table S6:**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	2.1379	0.2035	10.507	2e-16
0 °C	-1.5362	0.2376	-6.467	1e-10
4 °C	-0.3250	0.2669	-1.218	0.223
8 °C	-0.1439	0.2839	-0.507	0.612
10 °C	-0.1278	0.2862	-0.446	0.655

Fixed-effects coefficients of a mixed-effect binomial model of the impact of chilling temperature on survival in *Aedes aegypti* (10 observations, 2 repeats, 6 degrees of freedom).

**Table S7:**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	2.6813	0.6348	4.224	2.40e-05
0 °C	-4.7689	0.7901	-6.036	1.58e-09
4 °C	-4.3281	0.7340	-5.896	3.71e-09
8 °C	-1.7908	0.6905	-2.593	0.0095
10 °C	-0.2607	0.7988	-0.326	0.7442

Fixed-effects coefficients of a mixed-effect binomial model of the impact of chilling temperature on full insemination rate in *Aedes aegypti* (25 observations, 5 repeats, 6 degrees of freedom).

**Table S8:**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	3.4639	0.3279	10.565	2e-16
5 g	-0.7809	0.3924	-1.990	0.04662
15 g	-0.8333	0.4077	-2.044	0.04095
25 g	-1.1701	0.3774	-3.101	0.00193
50 g	-2.3055	0.3527	-6.537	6.28e-11

Fixed-effects coefficients of a mixed-effect binomial model of the impact of compaction on survival in *Aedes aegypti* (10 observations, 2 repeats, 6 degrees of freedom).

**Table S9:**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	1.6917	0.4113	4.113	3.91e-05
5 g	-0.8650	0.5214	-1.659	0.097114
15 g	-1.9430	0.5038	-3.857	0.000115
25 g	-2.3848	0.5128	-4.651	3.30e-06
50 g	-4.3997	0.7244	-6.074	1.25e-09

Fixed-effects coefficients of a mixed-effect Binomial model of the impact of compaction on full insemination rate in *Aedes aegypti* (25 observations, 5 repeats, 6 degrees of freedom).

**Table S10:**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	1.1451	0.1941	5.901	3.62e-09
20 Gy	-0.3039	0.2610	-1.165	0.24420
40 Gy	-0.6813	0.2537	-2.685	0.00724
80 Gy	-1.2334	0.2591	-4.760	1.93e-06
100 Gy	-2.0553	0.2831	-7.259	3.89e-13

Fixed-effects coefficients of a mixed-effect binomial model of the impact of irradiation dose on the escape rate from the flight organ in *Aedes albopictus* (10 observations, 2 repeats, 6 degrees of freedom).

**Table S11:**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	1.94591	0.29650	6.563	5.28e-11
30 Gy	-0.09633	0.41301	-0.233	0.815573
90 Gy	-0.95266	0.36550	-2.606	0.009149
110 Gy	-1.07949	0.35539	-3.037	0.002386
150 Gy	-1.15745	0.34675	-3.338	0.000844

Fixed-effects coefficients of a mixed-effect binomial model of the impact of irradiation dose on the escape rate from the flight organ in *Aedes aegypti* (10 observations, 2 repeats, 6 degrees of freedom).

**Table S12:**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	1.7723	0.2121	8.355	2e-16
0 °C	-0.1790	0.3052	-0.587	0.55743
4 °C	-0.5881	0.2772	-2.121	0.03389
8 °C	-0.7465	0.2842	-2.627	0.00861
10 °C	-4.2738	0.3422	-12.488	2e-16

Fixed-effects coefficients of a mixed-effect binomial model of the impact of chilling temperature on the escape rate from the flight organ in *Aedes aegypti* (10 observations, 2 repeats, 6 degrees of freedom).



**Table S13:**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	1.2797	0.2076	6.164	7.08e-10
5 g	-0.5220	0.2602	-2.006	0.0449
15 g	-1.0462	0.2421	-4.322	1.55e-05
25 g	-1.2934	0.2360	-5.480	4.25e-08
50 g	-1.6801	0.2446	-6.868	6.52e-12

Fixed-effects coefficients of a mixed-effect binomial model of the impact of compaction on the escape rate from the flight organ in *Aedes aegypti* (10 observations, 2 repeats, 6 degrees of freedom).

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## Chapter 5

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# A rapid quality control test to foster the development of the Sterile Insect Technique against *Anopheles* *arabiensis*

The results presented in this chapter have been published as the manuscript:

Culbert N, Somda NSB, Hamidou M, Soma DD, Caravantes C, Wallner T et al.. A rapid quality control test to foster the development of the sterile insect technique against *Anopheles arabiensis*. Malar J. 2020;19: 44

## Abstract

With the fight against malaria reportedly stalling there is an urgent demand for alternative and sustainable control measures. As the sterile insect technique (SIT) edges closer to becoming a viable complementary tool in mosquito control, it will be necessary to find standardised techniques of assessing male quality throughout the production system and post-irradiation handling. Flight ability is known to be a direct marker of insect quality. A new version of the reference International Atomic Energy Agency/ Food and Agricultural Organization (IAEA/FAO) flight test device (FTD), modified to measure the flight ability and in turn quality of male *Anopheles arabiensis* within a two-hour period via a series of verification experiments is presented. *An. arabiensis* juveniles were mass reared in a rack and tray system. 7500 male pupae were sexed under a stereomicroscope (2500 per treatment). Stress treatments included irradiation (with 50, 90, 120 or 160 Gy, using a Gammacell 220), chilling (at 0, 4, 8 and 10 °C) and compaction weight (5, 15, 25, and 50 g). Controls did not undergo any stress treatment. Three days post-emergence, adult males were subjected to either chilling or compaction (or were previously irradiated at pupal stage), after which two repeats (100 males) from each treatment and control group were placed in a FTD to measure flight ability. Additionally, one male was caged with 10 virgin females for 4 days to assess mating capacity (five repeats). Survival was monitored daily for a period of 15 days on remaining adults (two repeats). Flight ability results accurately predicted male quality following irradiation, with the first significant difference occurring at an irradiation dose of 90 Gy, a result which was reflected in both survival and insemination rates. A weight of 5 g or more significantly reduced flight ability and insemination rate, with survival appearing less sensitive and not significantly impacted until a weight of 15 g was imposed. Flight ability was significantly reduced after

treatments at 4 °C with the insemination rate more sensitive to chilling with survival again less sensitive (8 and 0 °C respectively). The reported results conclude that the output of a short flight ability test, adapted from the previously tested *Aedes* FTD, is an accurate indicator of male mosquito quality and could be a useful tool for the development of the SIT against *Anopheles arabiensis*.

## Introduction

Malaria is a disease that still accounts for almost half a million deaths each year. The latest report published by the World Health Organization (WHO) on malaria highlighted that although fewer deaths were reported in 2017, the success in global malaria control has stalled [1]. Such statistics underline the urgency for alternative complementary control measures. The sterile insect technique (SIT) is one of several genetic control measures routinely used throughout the world to suppress, contain or eradicate various species of agricultural, veterinary or human insect pests [2, 3, 4]. A technique which has proved successful and sustainable, particularly when deployed as part of an area-wide integrated pest management programme (AW-IPM).

Despite strong efforts in the 1970s and 80s to roll out the technique against mosquitoes, one of the most devastating vectors of human disease, efforts were more or less abandoned until recent years. A build-up of insecticide resistance coupled with the global spread of species such as *Aedes aegypti* and *Aedes albopictus* has reignited interest in developing mosquito SIT as part of an AW-IPM approach, with the technique progressing towards implementation at an operational level. Mass-rearing technology has been standardised [5, 6, 7] and guidelines created for key vectors including *Anopheles arabiensis* [8], *Ae. aegypti* and *Ae. albopictus* [9]. More recently, research has arisen addressing some key elements of the handling, transport and release aspects of a mosquito SIT programme. Optimal transportation conditions have been advised for *An. arabiensis* [10] in addition to a study investigating various release conditions for both *An. arabiensis* and *Ae. aegypti* [11].

The ability of an insect to perform flight is known to be a direct marker of their quality [12]. Poor fliers will be unable to complete their primary goal which is to seek out and mate with wild females. Thus, it is imperative that in any SIT programme, insects released are of adequate quality. Quality control (QC) checks must be carried out routinely throughout critical steps of the mass-rearing process and both pre and post-release. Flight cylinders that can gauge flight ability have consistently been reported to be an accurate indicator of mating competitiveness and are routinely used as a QC tool in fruit fly, tsetse fly and moth SIT programmes [13, 14]. Such cylinders offer an easy to use, portable and rapid means of estimating sterile insect quality both pre and post-release.

Until recently, flight mills were the only method of assessing flight ability. Such tools require a high level of skill and the process of the flight test can be lengthy. The first mosquito QC tool was reported by Balestrino et al [15] which measured the flight ability of recently emerged *Ae. albopictus* from pupae that had been placed in 1 of 100 individual wells. Despite promising results, the test itself required a period of 48 to 72 hours to complete. However, the basis of this device was used to create a new flight cylinder that could measure the flight ability of 100 adult *Ae. aegypti* or *Ae. albopictus* mosquitoes within a 2-hour period. A series of 10 prototypes were created and verified by a series of stress treatments such as irradiation, chilling temperature and compaction [16]. The final device is now considered as the IAEA reference QC test for *Aedes* mosquitoes.

In order to expand the applicability of this device to *An. arabiensis*, it was necessary to first modify the diameter of the individual flight tubes. Initial experiments in the

*Aedes* device with male *An. arabiensis* were unsuccessful as their larger body size prevented them from completing vertical flight to escape the tubes. A new prototype FTD was constructed in exactly the same manner as with the first version for male *Aedes* mosquitoes with one simple adaptation; the diameter of each individual flight tube was increased from 8 to 10 mm. Preliminary experiments with the new prototype proved successful and thus a series of 10 new FTDs were constructed in order to validate the flight ability test as an indicator of overall quality via a series of stress treatment experiments as was done when validating the initial FTD for male *Ae. aegypti* and *Ae. albopictus*. Male *An. arabiensis* were exposed to a range of irradiation doses, chilling temperatures and levels of compaction and subsequent flight ability measure. To further link flight ability to quality, male survival and mating capacity were also investigated as reference tests.

## **Materials and Methods**

### **Mosquito colony source and mass rearing procedure**

All experiments were undertaken using the Dongola strain of *Anopheles arabiensis* sourced from the Northern State of Sudan (Tropical Medicine Research Institute, Khartoum). The laboratory colony was maintained at the Insect Pest Control Laboratory (IPCL) of the joint Food and Agricultural Organisation/International Atomic Energy Agency (FAO/IAEA) Division of Nuclear Techniques and Agriculture, Seibersdorf, Austria since 2005. Larvae were mass-reared in plastic trays held in a mechanized stainless-steel rack developed at the IPCL [6]. Eggs were quantified using the method defined in Maiga et al [7] wherein 4000 eggs are added to each tray, containing 4 l of deionised water, within a plastic ring which floats on the water surface. Water was added to each tray 24 h before the addition of the eggs to

allow the temperature of the water to acclimatize to that of the ambient air temperature. Larvae were fed daily on a 1% (wt/vol) diet developed by the IAEA and described in [17], following the feeding regime described in the guidelines for mass rearing *Anopheles* mosquitoes [18]. Larvae were maintained in a large climate-controlled room (88 m<sup>2</sup>) with temperature and humidity maintained at  $30 \pm 1$  °C,  $70 \pm 10\%$  RH, respectively.

Adults were maintained under controlled temperature, RH and light regimes ( $27 \pm 1$  °C,  $70 \pm 10\%$  RH, 12:12 h light:dark (L:D) photoperiod with 1 h periods of simulated dawn and dusk). Eggs were generated and gathered for all experiments following the *An. arabiensis* mass-rearing guidelines developed at the IPCL [8]. Mosquitoes were housed in standard plastic rearing cages (30 × 30 × 30 cm – Bugdorm, Taipei, Taiwan) and provided with constant access to a 5% sugar solution until day 3 when experiments were performed. Mosquito maintenance and the age of the adults at the time all described experiments were performed were chosen to reflect what may occur in a mass rearing facility prior to a release of sterile males. Despite *An. arabiensis* males reaching sexual maturity within 48 hours of emergence, males younger than 3 days old display low insemination rates [19]. There were two replicates for each stress treatment in addition to two control samples for each experiment performed.

### **Irradiation procedure and experimental design**

On the first morning that pupation was noted (7 days after eggs were placed in the trays), the rack was tilted. Pupae and larvae were separated by adding them to an Erlenmeyer flask containing dechlorinated water and swirling to create a vortex. The



difference in buoyancy and swimming behaviour between the larvae and pupae, during which the pupae rise to the surface and the larvae swim downwards, allows an accurate separation of the two life stages [20]. The first observed pupae were discarded from the experiment and the larvae placed back into mass rearing trays. After a period of 2 hours, pupae were then separated from the larvae once more ensure that the age of the experimental pupae was between 0 and 2 hours. Pupae were handled using disposable transparent pipettes. Males were separated from females by distinguishing differences in their genitalia under a stereomicroscope while placed on a transparent petri dish lid [20]. Pupae were irradiated at  $24 \pm 2$  hours in batches of 250 inside a self-contained  $^{60}\text{Co}$  Gamma Cell 220 (Nordion Ltd, Kanata, Ontario, Canada). A dosimetry system using Gafchromic MD film was used to verify absorbed dose. A range of irradiation doses required to induce full sterility and beyond, and thus expected to reduce the quality of the adult males, were selected. Control males were taken to the Gamma Cell room but not placed inside or exposed to any irradiation dose whilst experimental males received a dose of 50, 90, 120 or 160 Gray (Gy).

### **Chilling procedure and experimental design**

Following on from previously reported research from IPCL [10], a range of chilling temperatures that were known to be tolerable (4, 8 and 10° C), were selected, A lower temperature of 0° C was chosen with the intention to reduce quality post-chilling as it was beyond the range of temperatures previously tested and found to be tolerable. On day 3, cages of 250 adult males were immobilised at each of the above temperatures for a period of two hours, with control males left under insectary conditions ( $27 \pm 1$  °C).

### **Compaction procedure and experimental design**

On day 3, batches of 250 adult male *An. arabiensis* were immobilised at 10 °C for a period of 2 hours. As noted above, 10° C has been found not to impact quality. During immobilisation, as described above, adult males were subject to varying levels of compaction by being placed under a mosquito substitute particle - cumin seeds. Cumin seeds were selected after analysing the morphological properties and weights of several substitute particles such as fennel, rice, anise and poppy seeds. Cumin seeds were found to best match the weight and characteristics of adult mosquitoes and were thus selected. 5, 15, 25 or 50 g of cumin seeds were weighed, wrapped in mesh and sealed with an elastic band corresponding to 0, 0.25, 0.76, 1.27 and 2.55 g/cm<sup>2</sup> respectively.

### **Assessing survival rate and mating propensity as a measure of quality**

The aim was to link post stress-treatment flight ability with survival rate and mating capacity which are known quality parameters. Firstly, survival rate was measured by removing and quantifying the number of dead adults in both experimental and control cages for 15 days post stress-treatment in both replicates. The number of adults available for the longevity experiment (N), after flight ability and mating capacity tests, ranged between 117-227 for the irradiation experiment. For the chilling and compaction experiments, N ranged between 106-149 and 77-112 respectively. Mating capacity was evaluated by placing a single control or experimental male from each treatment into a standard cage (30 x 30 x 30 cm) with 10 virgin females from the same cohort for a period of 5 days, with continued access to a 5% sugar solution. Females were then dissected and the spermatheca removed and viewed under a stereomicroscope for the presence or absence of sperm to clarify

the insemination rate. Five repetitions were carried out for both the control and experimental treatments.

### **Flight test device and experimental procedure**

A flight test device (FTD), created within the IPCL as a QC tool for *Aedes* mosquitoes by measuring their flight ability [16], was adapted to compensate for the slightly larger body size of *An. arabiensis* males. The modified FTD consists of 22 transparent acrylic plastic (Polymethyl methacrylate – PMAA) tubes which are in turn housed within a larger PMAA tube. Any remaining gaps between the tubes were filled with silicon. A final larger PMAA tube which creates a containment area after the males exit the flight tubes surrounds the inner two series of tubes (all dimensions remain as described by Culbert et al, 2018 [16] except for the individual flight tube internal diameter which increased from 8 to 10 mm). The flight ability test was conducted by mouth aspirating a sample of 100 male mosquitoes into the entry hole at the base of the FTD, with 2 repetitions for the control and each experimental group. Adults were allowed a period of 2 hours to escape following which, the number of mosquitoes remaining at the base of the FTD and those that had escaped were counted. Flight ability is determined by dividing the number of adults that had escaped the flight tubes by the total number that began the experiment.

### **Further assessment of the flight test device: effect of light and time to pupation on the flight ability**

It is known that wild *Anopheles* males are flight active mainly at the onset of dusk, especially around time of sunset for swarming/mating and/or sugar meal [21, 22].

To integrate the impact of daily activity pattern of *Anopheles* mosquitoes on the flight ability test, the flight ability of *An. arabiensis* was evaluated in two simulated light conditions: day light (390.2 Lux) and dusk (3.9 Lux). Mosquitoes were submitted to dusk conditions 30 min prior to the tests. For each light condition, flight tests were performed on 2 groups of mosquitoes separately: 1) Mosquitoes were chilled in a climatic chamber at 4 °C for 2h and then kept under insectary conditions ( $27 \pm 1$  °C,  $70 \pm 10\%$  RH) for 90 min prior to the test. Mosquitoes were from the first pupa collection day (day 7 from seeding the eggs). 2) Mosquitoes were irradiated at pupal stage ( $24 \pm 2$ h old) at 90 Gy and kept under insectary conditions ( $27 \pm 1$  °C,  $70 \pm 10\%$  RH) until tests were performed. Mosquitoes were from the second pupae collection day (day 8). A control group (not chilled and not irradiated) was used in each test. Tests were performed between 9-12 am for the day light conditions and between 4-7 pm for the dusk conditions. Mosquitoes were kept for 30min under dusk conditions prior to the flight tests. Three replicates were performed for all treatments (control, chilled, irradiated) in each light condition. Only the flight ability was measured. To assess the effect of time to pupation on the flight ability, data from the control groups (pupae collected on day 7 and those on day 8) were compared. Then, 6 replicates were considered for each control group. The data for control groups, chilled and irradiated groups were also compared to the results obtained for same treatments in former experiments to assess the repeatability of flight ability measures.

### **Statistical analysis**

To analyse the impact of the various stress treatments on day fifteen survival rates, insemination rates and escape rates from the flight test device (response variables),

binomial linear mixed effect models were used. Repetitions were treated as random effects and the treatment levels for irradiation, chilling and compaction were then used as fixed effects. The likelihood ratio test [23, 24] was used to ascertain the significance of the fixed effects. Binomial linear mixed effect models were also used to determine if the escape rate could be used to predict the other QC parameters (day fifteen survival rates and insemination rates). This was achieved by using the QC parameters as response variables and the escape rate as a fixed effect. To describe the proportion of variance explained by the model between the observed and predicted values, the  $R^2$  (coefficient of determination) was then used [25, 26]. P-values and fixed-effects coefficients of all models used for data analysis can be found in Tables S1-S9. To analyse the data from light intensity and time to pupation experiments, general linear mixed models were used with the treatments (chilling, irradiation, light condition, time to pupation) as fixed effects, the flight ability as response variable and replicates as random effects. The outputs can be found in Tables S10-S12.

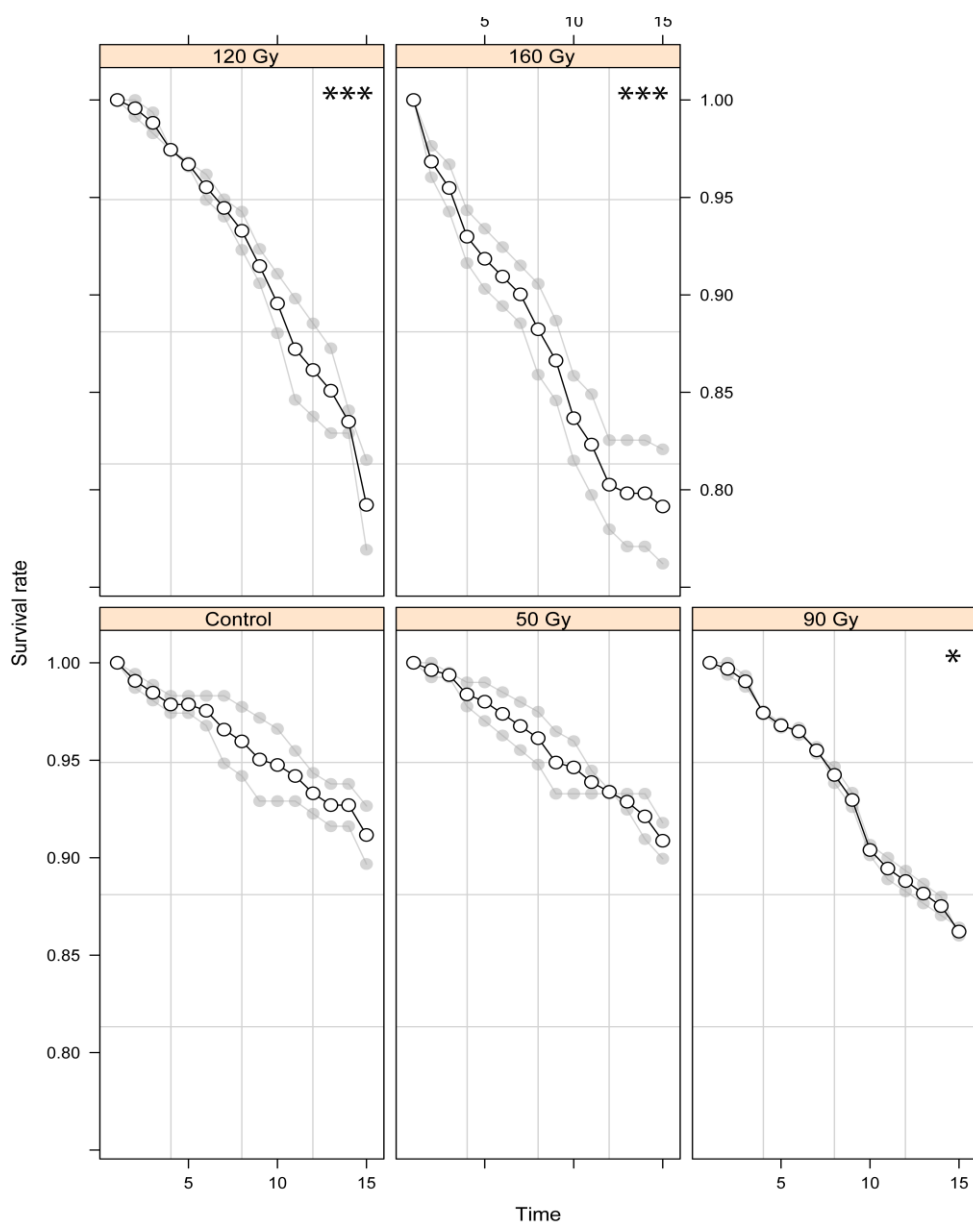
## **Results**

### **Impact of treatments on survival and insemination rates**

The survival and insemination rate of male *Anopheles arabiensis* was significantly reduced at a dose of 90 Gy or more (Figs. 1-2, Table S1 and S4,  $P = 0.043$ ,  $P = 0.016$ ). Interestingly, only exposure to the lowest chilling temperature (0°C) significantly reduced survival (Fig. S1, Table S2,  $P = 0.006$ ), however, exposure to 8 °C or less caused a significant decrease in the insemination rate (Fig. 2, Table S5,  $P = 0.030$ ). A compaction weight of 15 g was found to significantly decrease survival (Fig. S2, Table

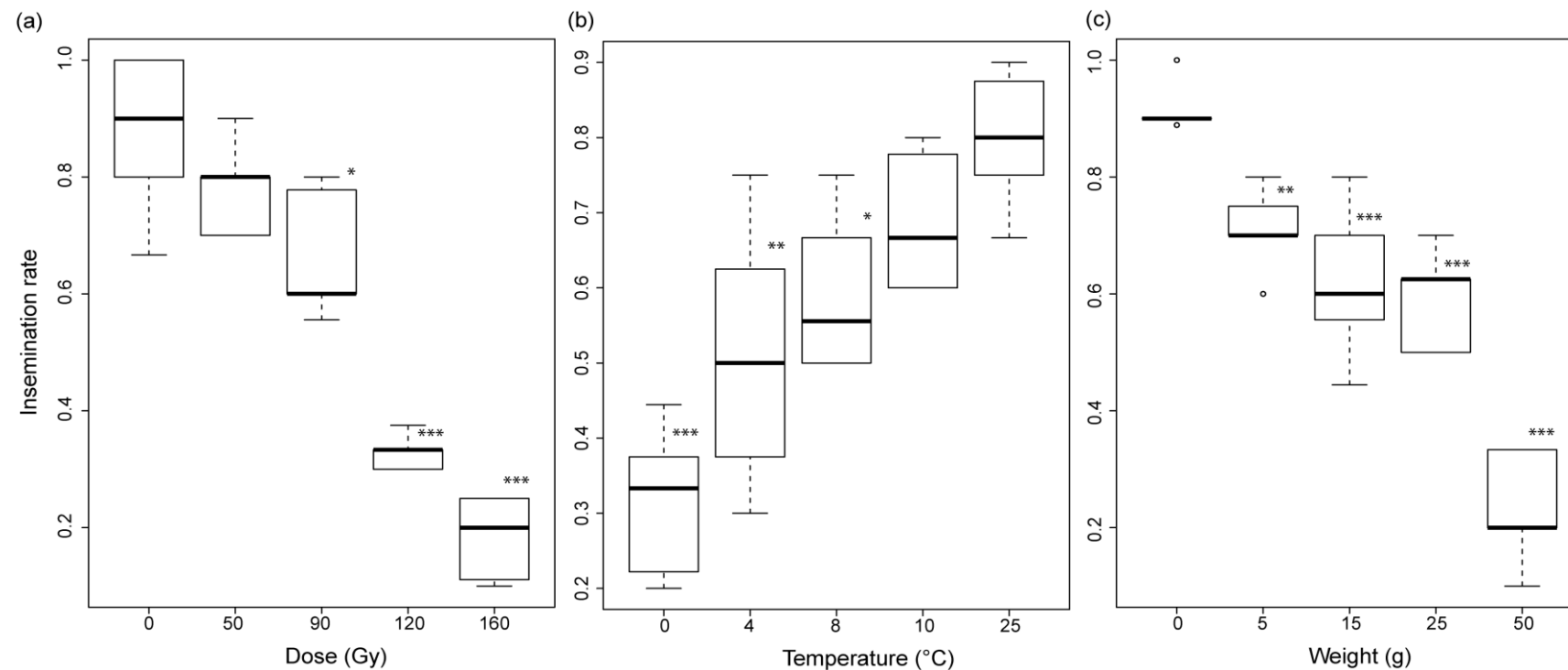
S3,  $P = 0.003$ ), whilst a weight equal to 5g or more caused a significant impact upon the insemination rate (Fig. 2, Table S6,  $P = 0.012$ ).

**Figure 1:**



The survival rates of male *Anopheles arabiensis* subject to a range of irradiation doses for 15 days. Significant differences between the control group (no irradiation) and treatment groups (50, 90, 120 and 160 Gy) are represented as follows (\* $p < 0.05$ , \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). Individual values of the various replicates are indicated in light grey and mean values shown as a solid line

**Figure 2:**



The insemination rates of male *Anopheles arabiensis* males subject to a range of stress treatments. Panel (a) represents the impact of various irradiation doses, panel (b) represents the impact of chilling temperature and panel (c) various compaction weights. The median value and the quartiles, horizontal bars the 95% percentiles and dots the minimal and maximal values are shown in each Boxplot with significant differences between treatment groups and the control group denoted as follows (\* $p < 0.05$ , \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

### Impact of treatments on flight ability

The results given show that the flight ability of male *An. arabiensis* is harmed by conditions of a 90G y dose or above, 4 °C and below and compaction of 5g (Fig. 3, Tables S7-9,  $P < 0.001$ ,  $P = 0.007$ ,  $P = 0.009$ ). Overall, results showed that flight ability is an accurate parameter of QC as it appeared to be sensitive to each of the various stress treatments imposed upon male *An. arabiensis* (Fig. 4, Table 1). It explained 48 – 98% of the variance of survival rates and 72 - 90% of the variance observed in insemination rates depending on the stress treatment considered. All three QC parameters significantly decreased at a dose of 90 Gy and above. Flight ability and insemination rates were most sensitive to excessive compaction (5 g). Flight ability was less sensitive to chilling (4 °C) in comparison to insemination rate (8 °C) but more sensitive than survival (0 °C).

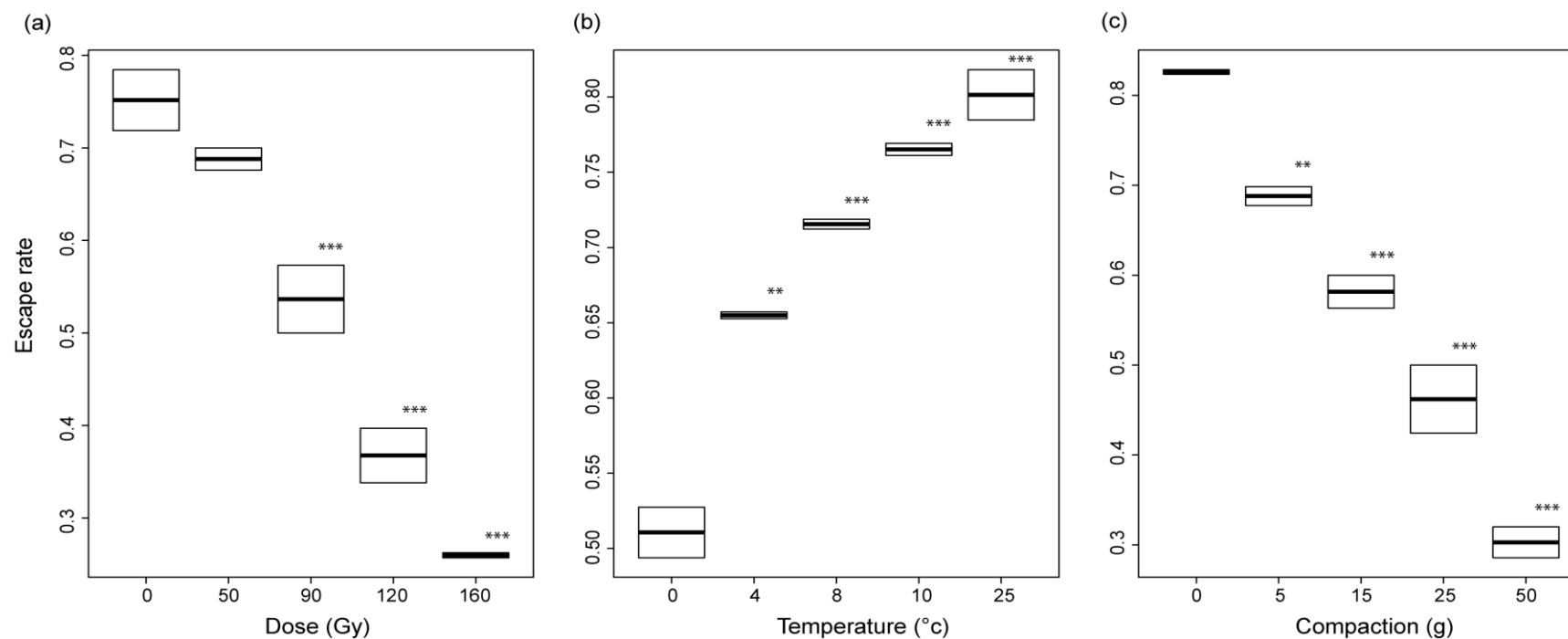
**Table 1:**

Treatment	First significant impact on escape rate	First significant impact on survival rate at day 15	First significant impact on insemination rate
Irradiation	90Gy	90Gy (0.75***)	90Gy (0.90***)
Chilling	4°C	0°C(0.48**)	8°C(0.72***)
Compaction	5g	15g(0.98***)	5g(0.88***)

The first values of the different treatments significantly impacting each male quality indicator are presented. The values in brackets correspond to the proportion of explained variance (r-square), used as a model quality indicator, based on a linear mixed-effect model where the response variable (survival, insemination and full insemination rates) is predicted using the escape rate as a fix effect and the repeats as random effects.. (\*  $0.05 > P > 0.02$ , \*\*  $0.01 > P > 0.001$ , \*\*\*  $P < 0.001$ )



**Figure 3:**



The escape rates of male *Anopheles arabiensis* mosquitoes subject to a range of stress treatments. Panel (a) represents the impact of various irradiation doses, panel (b) represents the impact of chilling temperature and panel (c) various compaction weights. The median value and the quartiles, horizontal bars the 95% percentiles and dots the minimal and maximal values are shown in each Boxplot with significant differences between treatment groups and the control group denoted as follows (\*p < 0.05, \*\*p < 0.01; \*\*\*p < 0.001).

**Figure 4:**

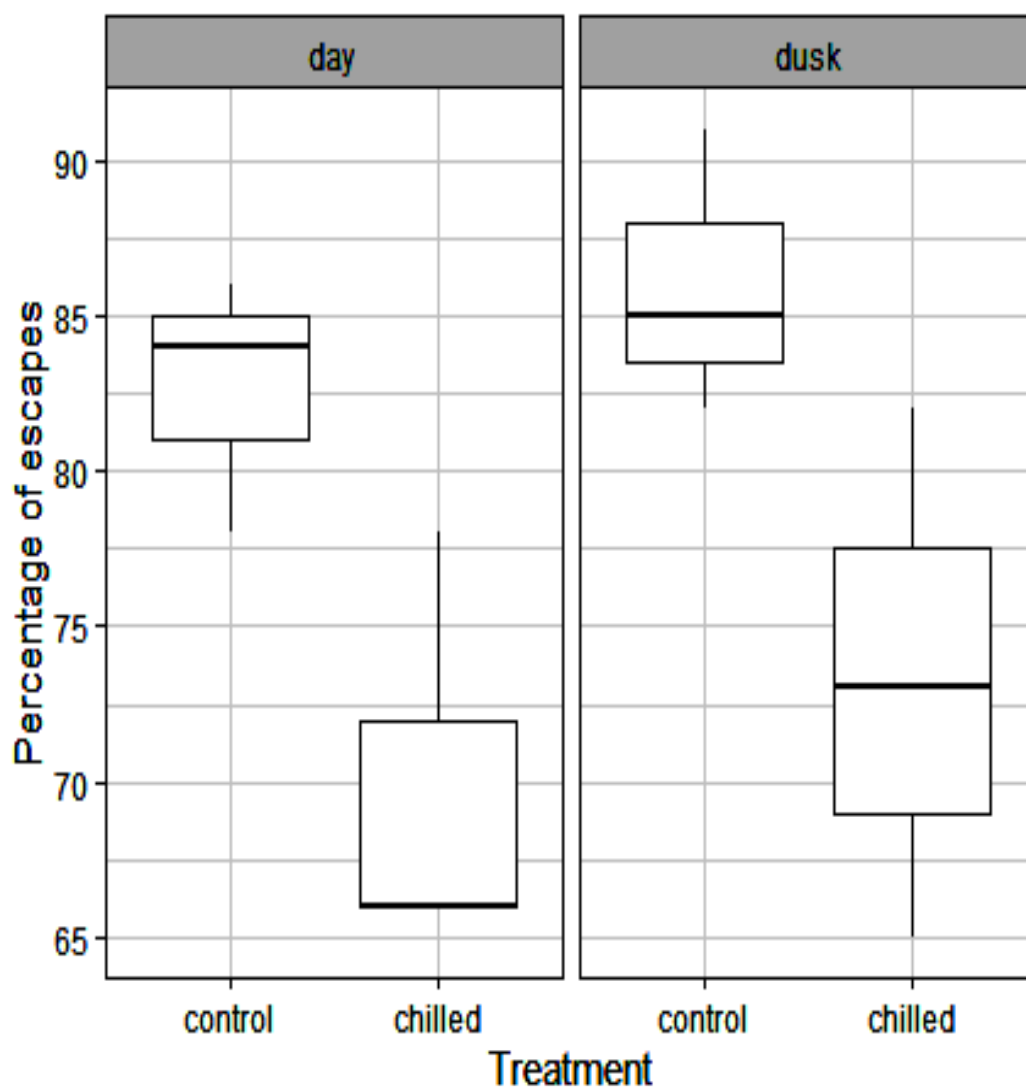


Male *Anopheles arabiensis* during a flight ability test.

Further assessment of the flight test device confirmed that male *An. arabiensis* flight ability was significantly reduced when chilled at 4 °C for 2 hours (Fig. 5, Table S10,  $P < 0.001$ ) or irradiated at 90 Gy (Fig. 6, Table S11,  $P = 0.0387$ ). However, the light did not impact the flight ability. Indeed, the best models excluded this factor. In addition, flight ability was not affected by the batch of pupa collection (first or second day of collection) (Table S12,  $P = 0.334$ ) Finally, the flight ability of chilled (4°C) and

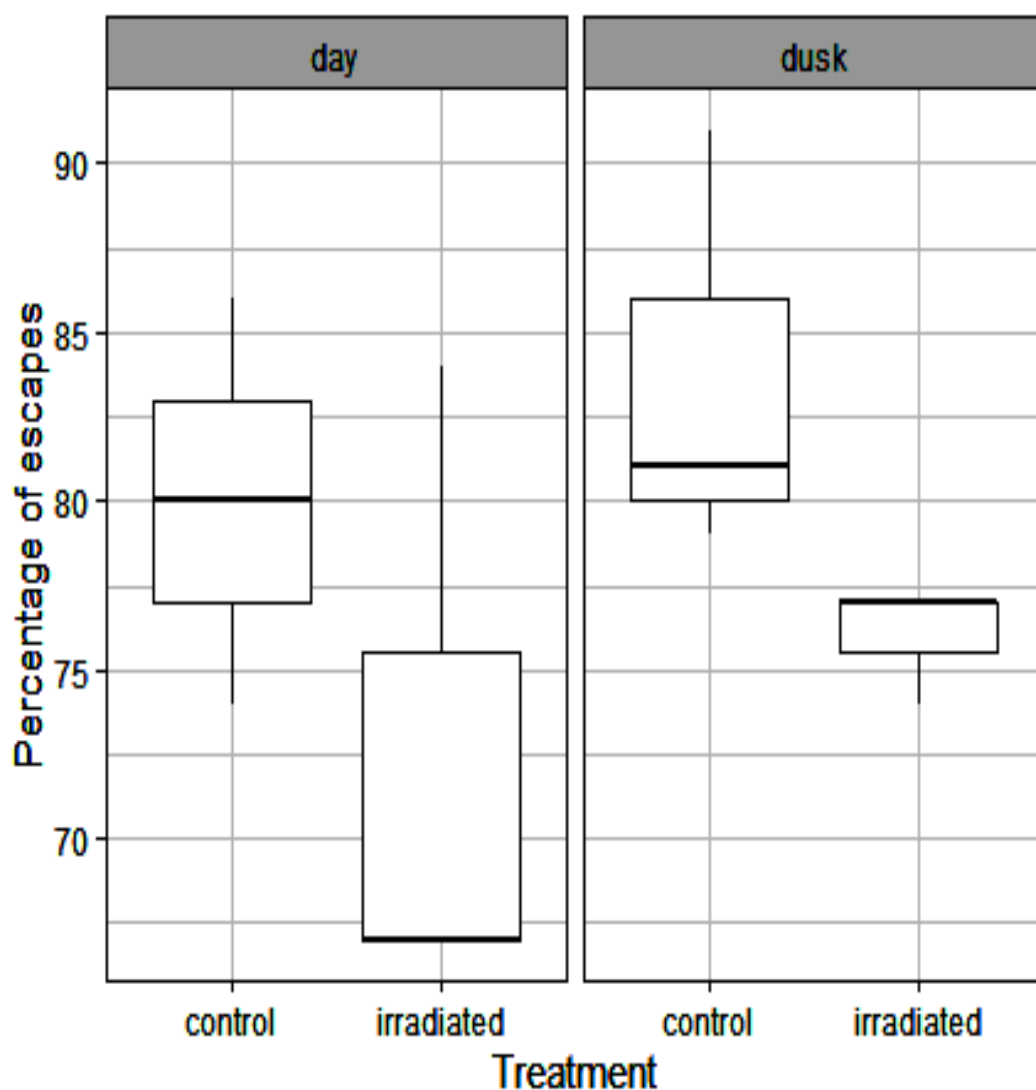
control groups were compared between the first and second experiments (different operators and experiment conducted one year later) and no significant difference was observed (the best model did not retain the experiment number). The same result was obtained when comparing the irradiated and control groups of the two experiments. This showed a good repeatability of the flight ability tests.

**Figure 5:**



The escape rates of male *Anopheles arabiensis* mosquitoes subject to chilling at 4°C in function of the light intensity.

**Figure 6:**



The escape rates of male *Anopheles arabiensis* mosquitoes subject to irradiation at 90 Gy in function of the light intensity.

## Discussion

In any release campaign, with a sterile component, it is imperative that the insects released are of adequate quality, with a maximal lifespan and minimal damage. Colonisation, mass rearing, irradiation and handling are just a few examples of factors that can reduce male quality. One method of estimating insect quality is to measure

their flight ability, a known reliable marker [12]. Following the development and verification of a novel flight test device to assess the quality of male *Ae. aegypti* and *Ae. albopictus* mosquitoes [16], a similar tool was created for male *An. arabiensis*.

The irradiation process is a crucial part of the SIT. It is critical to administer an irradiation dose that ensures a sufficient level of sterility without impacting subsequent survival and male quality, as it is well recognised that ionizing radiation can significantly decrease subsequent field competitiveness [27]. A dose of 90 Gy or more caused a significant decrease in flight ability, survival and insemination rates in *An. arabiensis* males. These results mirror those of an earlier study in which *Ae. aegypti* males exposed to 90 Gy or more displayed the same significantly reduced quality across all measured parameters [16]. It was postulated that administering a dose of 90 Gy, which falls between the partially (70 Gy) and fully sterile irradiation dose (120 Gy), to *An. arabiensis* males, would not cause a significant decrease in quality as previous studies have shown that they are still competitive even at a dose of 120 Gy [28]. As suggested previously, studies must be carried out in which male competitiveness is assessed either in semi-field or field conditions and directly compared to flight ability. It may be that flight ability offers a more accurate depiction of male mosquito quality than competitiveness measured in semi-field conditions and warrants further investigation.

Sterile insects are routinely released in a chilled state after being immobilised to facilitate the ease of their handling during their packing and transportation from the rearing facility to the release site. It seems logical to assume that a release campaign involving mosquitoes will involve a similar process as to what is currently used in

other insects including Mediterranean fruit flies [29] and tsetse flies [30] and thus understanding how chilling temperature impacts male quality is fundamental to a successful campaign. A previously reported study conducted within the IPCL laboratory [10], exposed male *An. arabiensis* to a range of chilling temperatures for various durations and concluded that only exposure to 2 °C, the coldest temperature tested, for 24 hours, the longest duration tested, significantly reduced survival in comparison to non-chilled control males. Therefore, it was suspected that only the lowest chilling temperature of 0 °C would significantly reduce survival. This was indeed found to be the case and, additionally, it reflects the findings of the initial verification experiments of the FTD created for *Ae. aegypti* males [16]. Despite the lesser effect of chilling temperature on male survival, regardless of species, flight ability and insemination rate are more sensitive. Significant decreases in insemination rate are apparent at 8 °C in both *An. arabiensis* and *Ae. aegypti*, whilst flight ability begins to decline at 4 and 8 °C respectively. The impact of chilling temperature upon male quality does appear to be species-specific and thus what seems appropriate for one should not automatically be inferred for another.

The absence of impact of light on flight ability may be attributed to the attractive effect of the BG lure (Biogents, Regensburg, Germany) used in the tests. In addition, this might be explained by the adaptation of the strain to the laboratory conditions where males do not need to join swarms to mate. The result indicates that the flight ability test can be performed at any time of the day and also show a good repeatability. Moreover, the similarity of males from the first and second days of pupa collection indicates that these groups of males might be similar in fitness and then both may be considered for releases in SIT programmes.

To ensure the cost-effectiveness of a mosquito SIT programme, it is necessary to determine the maximum number of mosquitoes that can be transported in any given container without impacting their quality, or in other words the maximum tolerable level of compaction. The lowest compaction weight (5 g) was found to significantly decrease both flight ability and insemination rate. This is consistent with an earlier study conducted within the IPCL laboratory where male *An. arabiensis*, immobilised under high and low levels of compaction, did not differ in survival but did demonstrate a significantly reduced survival compared to controls under no compaction [10]. It also echoes results in the initial verification studies of the FTD using male *Ae. aegypti* where flight ability was also found to be significantly reduced following compaction in excess of 5 g [16]. Interestingly, *An. arabiensis* survival only began to significantly decrease following 15 g of compaction. Although males may be surviving after compaction under this weight, their quality was reduced as shown by their inability to fly and thus inseminate females, as is reflected in the significantly lower survival and insemination rate observed after 5 g of compaction. This finding is in keeping with a study recently published wherein several quantities (10, 40 and 240) of male *Ae. aegypti* were compacted into volumes of 1 cm<sup>3</sup> and shipped across the USA [31]. Results indicated that survival was highest following the greatest level of compaction (240/ cm<sup>3</sup>) however, a higher degree of damage was observed on the adults post-shipping. Similar outcomes from both studies highlights that not only do male mosquitoes need to survive the various stressors they encounter until their ultimate release, they need to be of an adequate quality. Survival is fruitless if they are damaged and in turn are unable to fly and mate with wild females, further facilitating the need for urgent QC tools such as the FTD.

The results of this series of experiments to validate a novel QC tool adds further support for the original version reported last year [16]. Once again, a strong correlation between flight ability and overall male quality was noted. The FTD offers a rapid and easy way of gaining insight into the quality of sterile male mosquitoes and is currently the only known tool available with such capabilities. All technical drawings of the FTD for *An. arabiensis* and also *Ae. aegypti* and *albopictus*, to allow accurate reproduction of the device, are available on our website (<http://www-naweb.iaea.org/nafa/ipc/public/manuals-ipc.html>).

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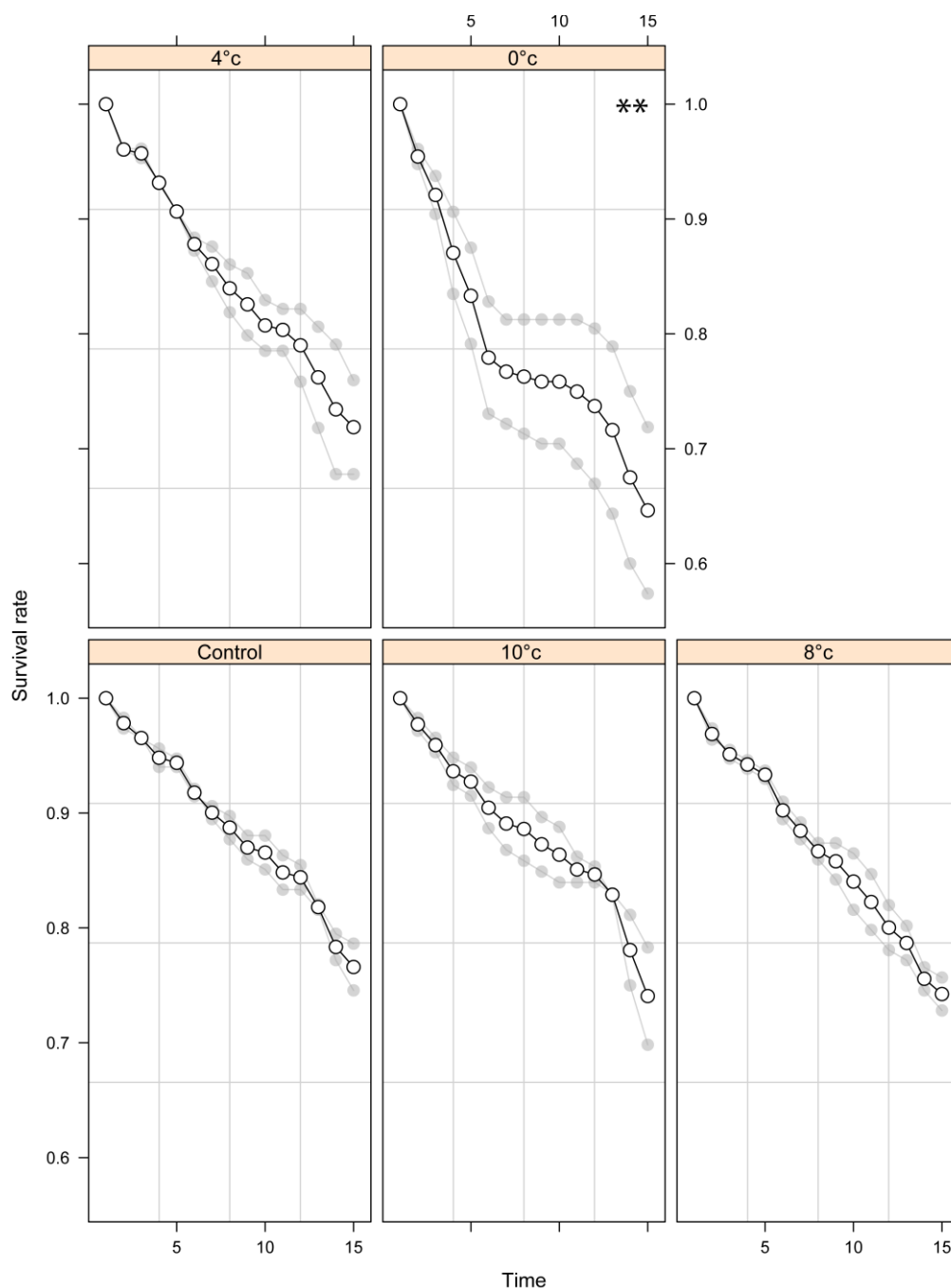
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## Supporting Information:

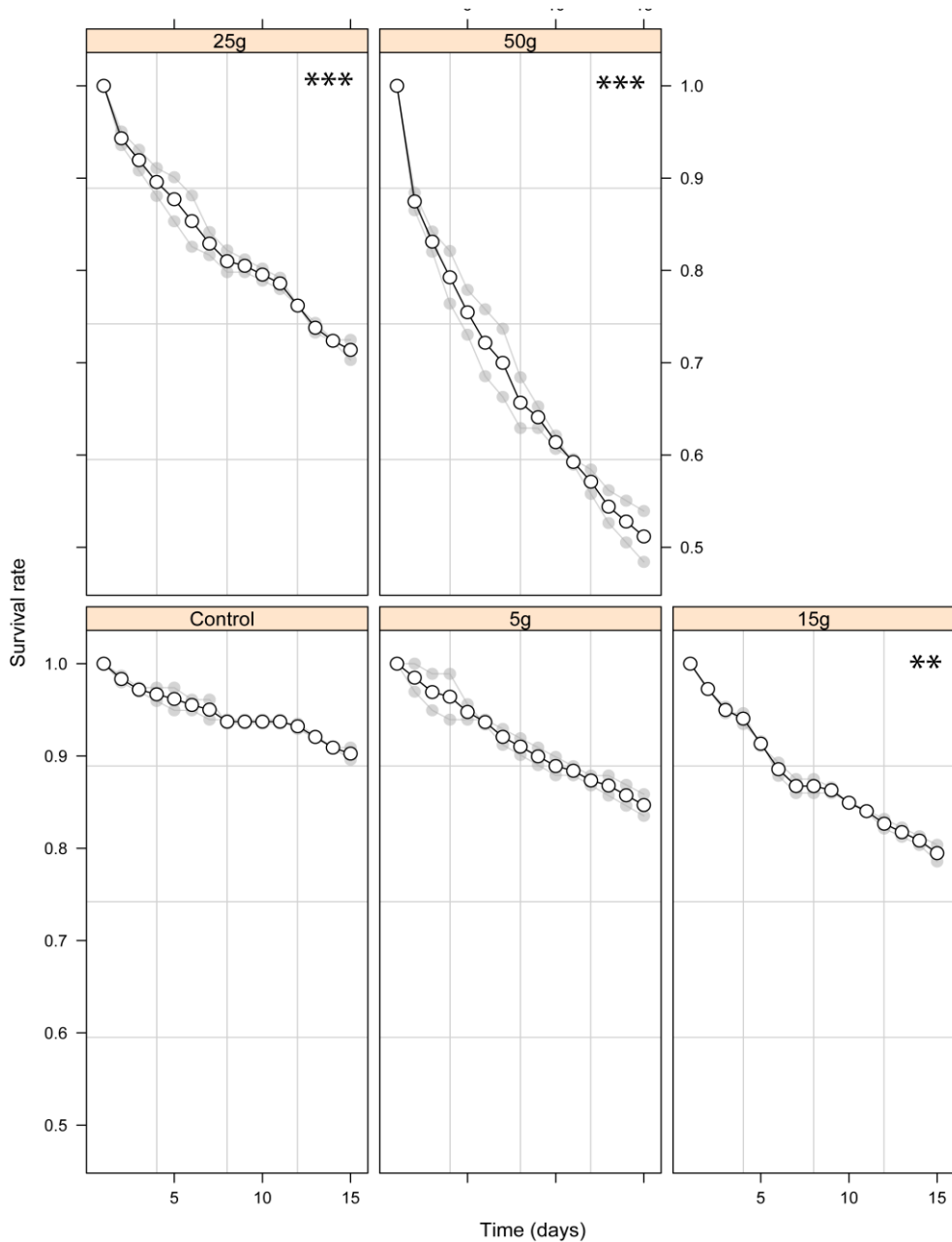
### Results

Figure S1:



The survival rates of male *Anopheles arabiensis* subject to various chilling temperatures for a period of 15 days. Significant differences between the control group (no chilling - 25°C) and treatment groups (10, 8, 4 and 0 °C) are represented as follows (\* $p < 0.05$ , \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). Individual values of the various replicates are indicated in light grey and mean values shown as a solid line.

**Figure S2:**



The survival rates of male *Anopheles arabiensis* exposed to a variety of compaction weights for a 15 day period. Significant differences between the control group (no compaction) and treatment groups (5, 15, 25 and 50 g) are represented as follows (\* $p < 0.05$ , \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). Individual values of the various replicates are indicated in light grey and mean values shown as a solid line.

## Tables:

**Table S1**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	2.3464	0.1944	12.071	2e-16
50 Gy	-0.0700	0.2708	-0.258	0.7961
90 Gy	-0.5129	0.2545	-2.016	0.0438
120 Gy	-0.9873	0.2454	-4.023	5.75e-05
160 Gy	-1.0189	0.2270	-4.488	7.18e-06

Fixed-effects coefficients of a mixed-effect binomial model of the impact of irradiation dose on survival in *Anopheles arabiensis*

**Table S2**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	1.1872	0.1555	7.636	2.23e-14
0 °C	-0.5672	0.2056	-2.759	0.00579
4 °C	-0.2633	0.2046	-1.287	0.19806
8 °C	-0.1296	0.2177	-0.595	0.55161
10 °C	-0.1477	0.2180	-0.678	0.49788

Fixed-effects coefficients of a mixed-effect binomial model of the impact of chilling temperature on survival in *Anopheles arabiensis*.

**Table S3**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	2.2357	0.2552	8.762	2e-16
5 g	-0.5216	0.3253	-1.603	0.10883
15 g	-0.8833	0.3051	-2.895	0.00379
25 g	-1.3194	0.2974	-4.436	9.14e-06
50 g	-2.1922	0.2947	-7.438	1.02e-13

Fixed-effects coefficients of a mixed-effect binomial model of the impact of compaction on survival in *Anopheles arabiensis*

**Table S4**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	1.9694	0.4358	4.519	6.21e-06
50 Gy	-0.7038	0.5536	-1.271	0.2036
90 Gy	-1.2763	0.5326	-2.396	0.0166
120 Gy	-2.6954	0.5374	-5.015	5.30e-07
160 Gy	-3.5009	0.5848	-5.987	2.14e-09

Fixed-effects coefficients of a mixed-effect binomial model of the impact of irradiation dose on insemination rate in *Anopheles arabiensis*

**Table S5**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	1.3863	0.3727	3.720	0.000199
0 °C	-2.1812	0.4925	-4.429	9.48e-06
4 °C	-1.3863	0.4794	-2.892	0.003829
8 °C	-1.0349	0.4781	-2.165	0.030410
10 °C	-0.5978	0.4857	-1.231	0.218324

Fixed-effects coefficients of a mixed-effect binomial model of the impact of chilling temperature on insemination rate in *Anopheles arabiensis*

**Table S6**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	2.4204	0.5217	4.639	3.50e-06
5 g	-1.5331	0.6108	-2.510	0.012074
15 g	-1.9095	0.6009	-3.178	0.001485
25 g	-2.0690	0.6016	-3.439	0.000583
50 g	-3.6334	0.6246	-5.817	5.99e-09

Fixed-effects coefficients of a mixed-effect binomial model of the impact of compaction on insemination rate in *Anopheles arabiensis*

**Table. S7**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	1.1090	0.2039	5.440	5.33e-08
50 Gy	-0.3185	0.2731	-1.166	0.243625
90 Gy	-0.9526	0.2648	-3.598	0.000321
120 Gy	-1.6513	0.2705	-6.104	1.04e-09
160 Gy	-2.1549	0.2761	-7.806	5.90e-15

Fixed-effects coefficients of a mixed-effect binomial model of the impact of irradiation dose on the escape rate from the flight organ in *Anopheles arabiensis*

**Table. S8**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	1.3959	0.2191	6.372	1.86e-10
0 °C	-1.3493	0.2669	-0.5055	4.30e-07
4 °C	-0.7551	0.2813	-2.684	0.00727
8 °C	-0.4745	0.2895	-1.639	0.10128
10 °C	-0.2147	0.3002	-0.715	0.47449

Fixed-effects coefficients of a mixed-effect binomial model of the impact of chilling temperature on the escape rate from the flight organ in *Anopheles arabiensis*

**Table. S9**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	1.5581	0.2246	6.938	3.98e-12
5 g	-0.7674	0.2962	-2.591	0.00957
15 g	-1.2264	0.2804	-4.374	1.22e-05
25 g	-1.7012	0.2813	-6.047	1.48e-09
50 g	-2.3911	0.3135	-7.628	2.39e-14

Fixed-effects coefficients of a mixed-effect binomial model of the impact of compaction on the escape rate from the flight organ in *Anopheles arabiensis*

**Table S10**

	Estimate	Standard error	z value	Pr(> z )
(Intercept)	1.6418	0.1279	12.839	<2e-16 ***
Chilled mosquitoes	-0.7255	0.1647	-4.405	1.06e-05 ***

Effect of chilling on male *Anopheles arabiensis* flight ability. Reference level for regression: Control (Not chilled mosquitoes). Significance codes : 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05. The best model excluded the light condition which did not affect the flight ability

**Table S11**

	Estimate	Standard error	z value	Pr(> z )
(Intercept)	1.4617	0.1356	10.780	<2e-16 ***
Irradiated mosquitoes	-0.4025	0.1947	-2.068	0.0387 *

Effect of irradiation on male *Anopheles arabiensis* flight ability. Reference level for regression: Control (Not irradiated mosquitoes). Significance codes : 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05. The best model excluded the light condition which did not affect the flight ability

**Table S12**

	Estimate	Standard error	z value	Pr(> z )
(Intercept)	1.6418	0.1279	12.839	<2e-16 ***
Second day of pupae collection	-0.1801	0.1864	-0.966	0.334

Effect of time to pupation on male *Anopheles arabiensis* flight ability. Reference level for regression: First day of pupa collection (Not irradiated mosquitoes). Significance codes : 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05



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## Chapter 6

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# A Standardised Method of Marking Male Mosquitoes for a Small-scale Release

The results presented in this chapter have been submitted as a manuscript to Parasites & Vectors. The manuscript was accepted on April 9th 2020.

## Abstract

Prior to a major release campaign of sterile insects, including the sterile insect technique, male mosquitoes must be marked and released (small scale) to determine key parameters including wild population abundance, dispersal and survival. Marking insects has been routinely carried out for over 100 years, however, there is no gold standard regarding the marking of specific disease-transmitting mosquitoes including *Anopheles arabiensis*, *Aedes aegypti* and *Aedes albopictus*. The research presented offers a novel dusting technique and optimal dust colour and quantities, suitable for small-scale releases, such as mark-release-recapture studies.

We sought to establish a suitable dust colour and quantity for batches of 100 male *An. arabiensis*, that was visible both by eye and under UV light, long lasting and did not negatively impact longevity. A set of lower dust weights were selected to conduct longevity experiments with both *Ae. aegypti* and *Ae. albopictus* to underpin the optimal dust weight. A further study assessed the potential of marked male *An. arabiensis* to transfer their mark to undusted males and females.

The longevity of male *An. arabiensis* marked with various dust colours was not significantly reduced when compared to unmarked controls. Furthermore, the chosen dust quantity (5 mg) did not negatively impact longevity ( $P = 0.717$ ) and provided a long-lasting mark. Dust transfer was found to occur from marked *An. arabiensis* males to unmarked males and females when left in close proximity. However, this was only noticeable when examining individuals under a stereomicroscope and thus deemed negligible. Overall, male *Ae. aegypti* and *Ae. albopictus* displayed a greater sensitivity to dusting. Only the lowest dust weight (0.5 mg) did not significantly reduce longevity

( $P = 0.888$ ) in *Ae. aegypti*, whilst the lowest two dust weights (0.5 and 0.75 mg) had no significant impact on longevity ( $P = 0.951$  and  $0.166$ ) respectively in *Ae. albopictus*.

We have devised a fast, inexpensive and simple marking method and provided recommended dust quantities for several major species of disease-causing mosquitoes. The novel technique provides an evenly distributed, long-lasting mark which is non-detrimental. Our results will be useful for future MRR studies, prior to a major release campaign.

## Introduction

Marking insects for scientific studies has been ongoing for almost 100 years [1, 2]. Mark-release-recapture (MRR) studies are extremely useful and allow calculations of dispersal and mortality rates as well as providing estimates of population size. Historically, MRR experiments have prodigiously focused on assessing female survival and dispersal, due to their significant role in disease transmission, as highlighted in a recent review [3]. A rekindled interest in male mosquito genetic control programmes, such as the sterile insect technique (SIT) [4], has seen the focus shift towards male ecology and highlighted the need for more male MRR studies, in particular for estimating the competitiveness of irradiated sterile males in the field, which is still missing in the literature for mosquitoes [5].

Fluorescent paints [6], dyes [7, 8] and dusts [9, 10] in an array of colours are commonly used, in addition to methods involving the use of radio isotopes [11], trace elements [12], protein immunomarking [13] and genetic or transgenic techniques, including mutations leading to a distinguishable phenotypic difference or transfection of a symbiont such as *Wolbachia* [14, 15, 16]. Currently, there is no universal marking method applicable to all insect species. The suitability of the marking method will depend on several criteria such as the species and number of insects required to be marked, the environment the insect will encounter, the nature of the experiment, ease of application, and ultimately the cost of the method. The chosen method of marking can exert different effects between different species.

A critical component of the sterile insect technique (SIT) package is being able to monitor sterile males post-release and to distinguish them from wild males when

collected in traps. Marking sterile insects prior to release is necessary to assess the efficiency of an SIT programme through a continuous assessment of the sterile to wild male ratio and is commonly achieved using MRR experiments. It is important to use a method of marking which is long-lasting, fast and easy to apply, in addition to being of low-cost, as within an operational level of an SIT programme, millions of sterile males may have to be marked at any one time. Moreover, the method of marking should have little or no effect upon the quality of the insect, with regard to competitiveness, flight ability and longevity.

Dusts or powders have been used to mark insects for more than 75 years [17] and are perhaps the most frequently used material [18]. The largest SIT programmes in the world involve rearing and releasing hundreds of millions of fruit flies on a weekly basis. For over five decades, fluorescent dust has been applied during the pupal stage, resulting in the emerging adults retaining their mark [19]. There are several types of fluorescent dusts that have been used in previous insect marking studies, from manufacturers such as Brilliant General Purpose [10], RADGLO [20] Brian Clegg [21] and DayGlo® [21]. DayGlo dusts are available in a broad spectrum of bright colours, allowing separate cohorts to be marked with different colours. Moreover, once applied, the dust is visible to the naked eye with enhanced detection under UV light. There are a variety of methods to apply dust to insects. Mosquitoes can be marked by placing them in a dusted plastic bag and gently shaking them. Previous studies that use a shaking procedure to mark delicate insects including mosquitoes have reported high mortality immediately after dusting in addition to coating them with too much dust [22]. Alternatively, a bulb duster can be used to puff dust on to the mosquitoes or a fan

placed within a cage to create a dust storm [21]. Mosquitoes are usually immobilized prior to dusting as this increases the likelihood of a more uniform coverage of dust.

Despite the large volume of publications dealing with marking insects, there is no gold standard available when it comes to marking specific species of mosquitoes. The research presented within this paper aims to develop a fast, low-cost and low-effort marking procedure for the small-scale release of *Anopheles arabiensis*, *Aedes aegypti* and *Aedes albopictus*, three of the main vectors of mosquito-borne diseases, that does not negatively impact the quality of the insect. A preliminary trial was conducted with male *An. arabiensis* using blue, yellow and pink DayGlo® dust to determine which colour provided the best visibility, both under UV light and with the naked eye. After selecting a dust colour, various weights of dust were applied to male *An. arabiensis* to determine the lowest dust amount necessary to mark a known number. The impact of this dust amount on the survival of male *An. arabiensis* mosquitoes and its persistence over time was investigated. It is vital that there is minimal or no transfer of the mark between released sterile males and the wild population in order to obtain accurate data during trapping. Thus, a further study investigating the transfer of dust between marked and unmarked control male and female *An. arabiensis* was conducted. Two further studies were undertaken to investigate a range of dust weights and their impact upon the survival of male *Ae. aegypti* and *Ae. albopictus* mosquitoes in addition to the persistence of the mark over time.

## **Materials and Methods**

### **Source of mosquito colonies and mass rearing procedures**

Experiments were carried out with mosquitoes from three established colonies.

*Anopheles arabiensis* (Dongola strain), were sourced from field collections in the Northern State of Sudan and transferred to the Food and Agricultural Organisation/ International Atomic Energy Agency (FAO/IAEA) Insect Pest Control Laboratory (IPCL) in Seibersdorf, Austria by the Tropical Medicine Research Institute in Khartoum in 2005. *Aedes albopictus* (Rimini strain) were sourced in Rimini, Italy and transferred to the IPCL by the Centro Agricoltura Ambiente “G. Nicoli” in Crevalcore, Italy in 2010. *Aedes aegypti* (Brazil strain) were sourced in Juazeiro, Brazil and transferred to the IPCL by Moscamed, Brazil in 2012. All strains have been subsequently maintained at the IPCL under controlled temperature, relative humidity (RH) and light regimes ( $27 \pm 1^{\circ}\text{C}$ ,  $70 \pm 10\%$  RH, 12:12 hour light:dark (L:D) photoperiod with 1 hour periods of simulated dawn and dusk). Eggs used for these experiments were generated following the *An. arabiensis* and *Aedes* rearing guidelines developed at the IPCL [23, 24]. *Anopheles arabiensis* larvae were mass-reared in plastic trays (100 x 60 x 3 cm) containing 4 litres of deionized water. Four thousand eggs were added per tray within a plastic ring floating on the water surface. Larvae were fed daily with 1% (wt/vol) IAEA diet developed and described in [25]. *Aedes* larvae were mass-reared in the same way as *An. arabiensis* larvae, but with 5 litres of water per tray and at a larval density of 18, 000 first-instar ( $L_1$ ) and provided with 7.5% IAEA diet as detailed in [26].

### **Pupae collection**

*Anopheles arabiensis* pupae were manually separated from larvae using a cold-water vortex technique as described in [27] and males separated from females by observing the terminalia under a stereomicroscope [28]. *Aedes* pupae were sexed mechanically using a Fay-Morlan glass plate separator [29] as redesigned by Focks (John

W. Hock Co., Gainesville, FL [30]). Male pupae were left to emerge inside 30 × 30 × 30 cm cages (BugDorm, Taipei, Taiwan) and provided with either a 5% (*An. arabiensis*) or 10% (*Aedes*) sucrose solution.

### **Dust colour, optimized dust quantity and marking technique**

The initial dust amounts tested with *An. arabiensis* were 1000 and 500 mg of dust per 100 male mosquitoes, based on the amounts used by [10] to mark *Culicoides* midges but these severely impacted immediate post-dusting survival. Therefore, a subsequent series of dust weights were investigated per 100 males and mortality assessed after 24 hours, i.e. 100, 75, 50, 15, 10, 7.5, 6.3 and 5 mg of dust, with the lowest dust amount (5 mg) chosen as the optimal weight for all subsequent experiments. This amount was chosen as it provided an even coating of dust that was visible with both the naked eye and under UV light. A lower series of dust weights were chosen for determining the optimal amount to use for *Ae. aegypti* and *albopictus* males, (1.5, 1, 0.75 and 0.5 mg per 100 males), as it was discovered during the first marking session that 5mg, despite marking adequately, left a surplus of dust behind. It was postulated that this may be due to their smaller body size as was noted when comparing the weight and volume occupied by 1000 males of all three aforementioned species in earlier laboratory tests, with both batches of male *Aedes* species weighing less than that of *An. arabiensis* (NJC personal observation). Both *Aedes* and *An. arabiensis* males were marked at 48 hours old.

Initially, three colours of fluorescent pigment were investigated - A-11 Aurora pink, A-17-N Saturn yellow and A-19 Horizon blue, all from the DayGlo® series as it is a brand routinely used to mark various other insects within the IPCL laboratory. Plastic



urine cups (x9 100 ml) were zeroed on an analytical balance and 5 mg of dust added to a cup, with 3 replicates per colour. After the addition of a plastic lid, the cups were shaken vigorously to coat the interior evenly. 12 batches of 100 male mosquitoes were immobilized at 4°C. All batches were transferred to the pre-dusted cups via a mouth aspirator. The cups were then gently rotated for 30 seconds, equating to 25 full rotations, to ensure all mosquitoes were evenly coated. The remaining 3 batches were rolled inside an undusted cup and served as controls. All males were returned to their original Bugdorms, maintained within the lab, whilst still inside their cups. The lid was removed, the cup placed on its side and the mosquitoes given sufficient time to recover before the cup was removed.

### **Marking and adult male longevity**

The impact of marking on male *An. arabiensis* longevity was assessed by comparing marked experimental with unmarked control males. Six batches of 100 male pupae were sexed under a stereomicroscope and allowed to emerge in Bugdorm cages with access to a sugar solution. All batches were immobilized and dusted as previously described. Three batches were marked with 5 mg of dust with the remaining 3 left undusted and serving as controls. Survival was monitored by removing dead individuals daily until all cages were empty on day 47. *Aedes* males were marked as described above for *An.arabiensis* but with 1.5, 1, 0.75 or 0.5 mg per 100 adults. Survival was monitored for 28 days post-dusting.

### **Dust persistence over time**

To investigate the persistence of dust over time in *An. arabiensis*, 3 groups of 500 males were immobilized and marked as previously described and released into large

field cages (1.8 m<sup>2</sup>) with a sugar solution provided. Two black plastic cups (500 ml) were placed inside each cage. One in a horizontal and one in a vertical position. Every second day, a lid was placed over each cup prior to removal to determine how many mosquitoes were inside. A photograph of each cup was taken to assess the persistence of the mark over time. All mosquitoes were then released into a fourth cage, to prevent resampling of the population, until few or no mosquitoes were collected in the black cups for several subsequent days.

### **Dust transfer between marked and unmarked adults**

Marked males were caged with unmarked males and females to determine whether they are capable of transferring dust. Pupae were sexed under a stereomicroscope into sets of 100 to populate 9 large Bugdorm cages (30 x 30 x 30cm) containing 100 of each sex. A further 9 sets of 100 males were sexed and allowed to emerge in small Bugdorm cages (15 x 15 x 15 cm). All cages contained a sugar solution. The 9 small cages of males were immobilized and dusted with 5 mg of dust as previously described. Each set of dusted males was then transferred to a large Bugdorm cage containing 100 undusted males and females. After 1 day, all marked males were then carefully aspirated out of 3 randomly selected cages before the cages were placed in a -20°C freezer to kill all remaining mosquitoes. Males and females were then screened under a stereomicroscope to check for the presence of dust particles. On day 3 post-dusting, this step was repeated with an additional 3 cages and again on day 7 with the remaining 3 cages.

### **Statistical analysis**

Binomial linear mixed effect models were used to analyse the impact of the various dust treatments on survival (response variable). The dust treatments were used as fixed effects whilst the replicates were set as random effects. The significance of fixed effects was tested using the likelihood ratio test [31, 32]. Fixed-effects coefficients of all models and their corresponding p-values are reported in Additional file 1 (Tables S1-S5).

## **Results**

### **Dust colour and optimised dust quantity**

The longevity of experimental *An. arabiensis* males did not significantly differ from undusted control males when dusted with blue ( $p = 0.217$ ), yellow ( $p = 0.804$ ) or pink ( $p = 0.335$ ) fluorescent dust (Table S1). A-11 Aurora pink was chosen as the dust colour for all further experiments as it was the most distinguishable colour under a UV microscope and with the naked eye. A dose of 5 mg for 100 males was selected as the optimal dust quantity for *An. arabiensis* after testing a range of dust (blue, yellow or pink) quantities in a preliminary study (Table 1).

### **Longevity of dusted adult males and dust persistence over time**

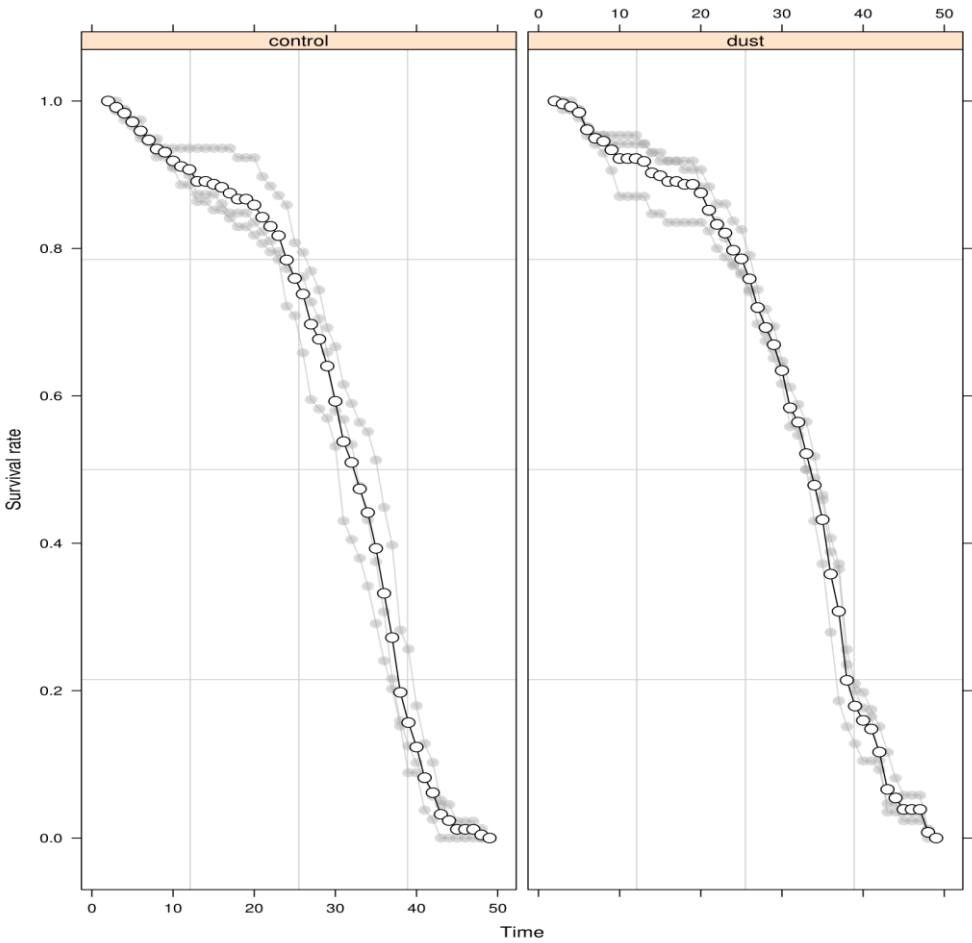
The longevity of dusted *An. arabiensis* males (5 mg/ 100 males) was not significantly different from that of undusted controls ( $p = 0.717$ , Figure 1, Table S2), with both control and dusted males surviving up to day 48. Photographs taken every second day showed that the selected dust amount (5 mg/100 adults) remained visible on marked males for upwards of one month (Figure 2).

**Table 1: Dust coverage and mortality 24 hours after dusting 100 male *Anopheles arabiensis***

Dust Amount (mg)	Mortality	Dust Coverage
1000	High	Very Heavy
500	High	Very Heavy
100	High	Very Heavy
75	High	Very Heavy
50	High	Very Heavy
15	Medium	Heavy
10	Medium	Heavy
7.5	Medium	Medium
6.3	Medium	Medium
5	Low	Good

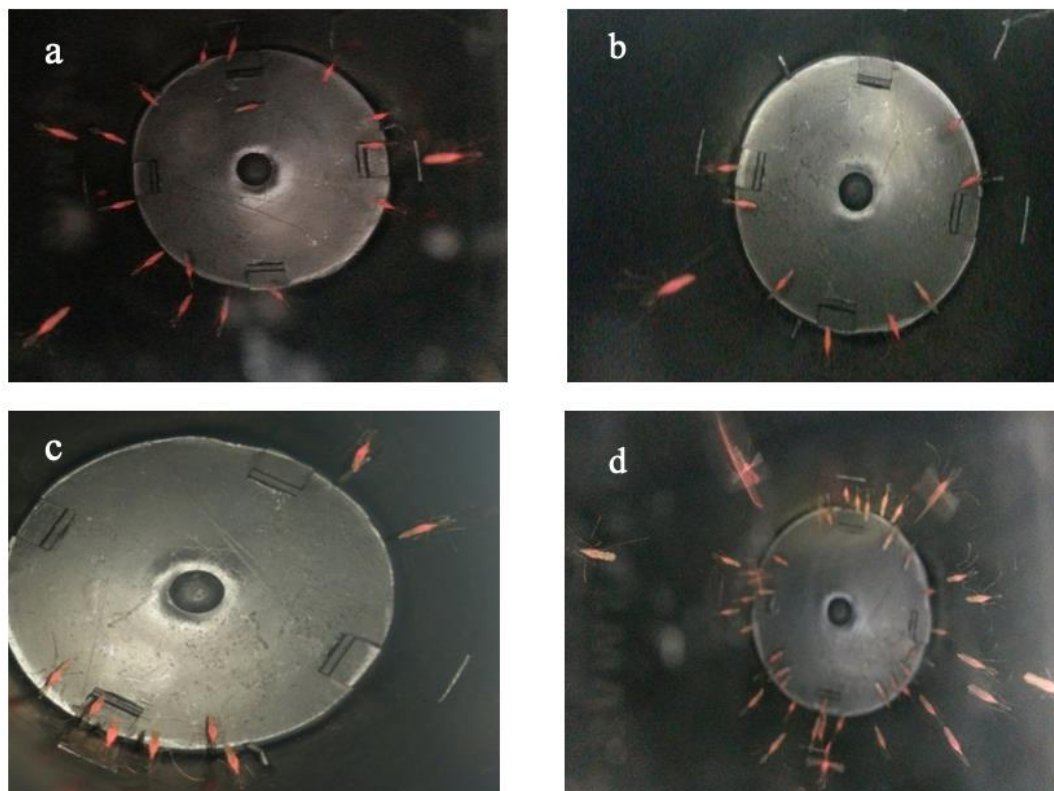
High > 50% mortality, medium > 10 < 50%, low < 10%.

**Figure 1:**



Survival was assessed for 5 mg of pink fluorescent dust for 100 males. Individual values of the replicates are indicated in light grey and mean values as a solid line

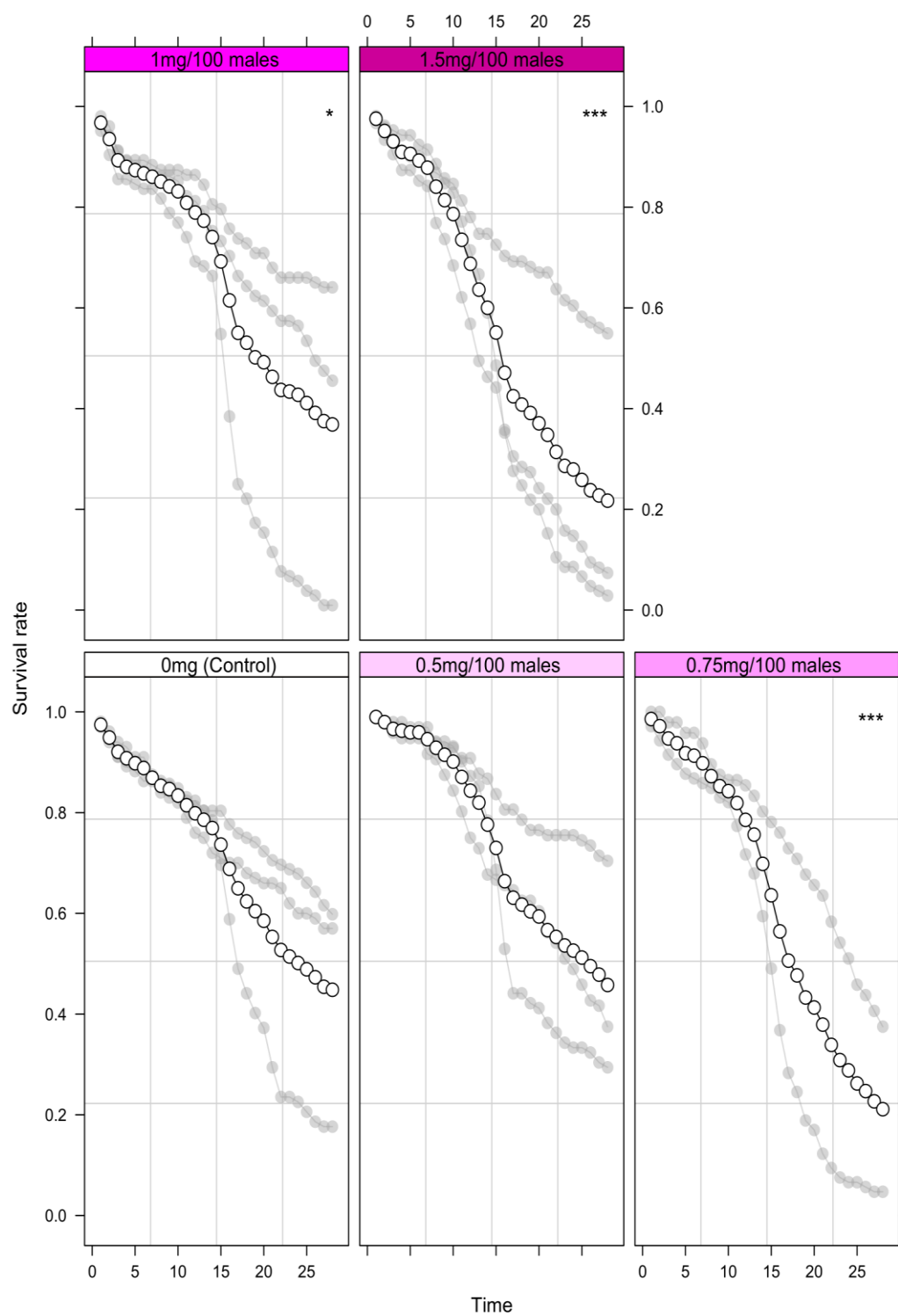
**Figure 2:**



a – 2 days, b – 8 days, c – 12 days and d – 28 days.

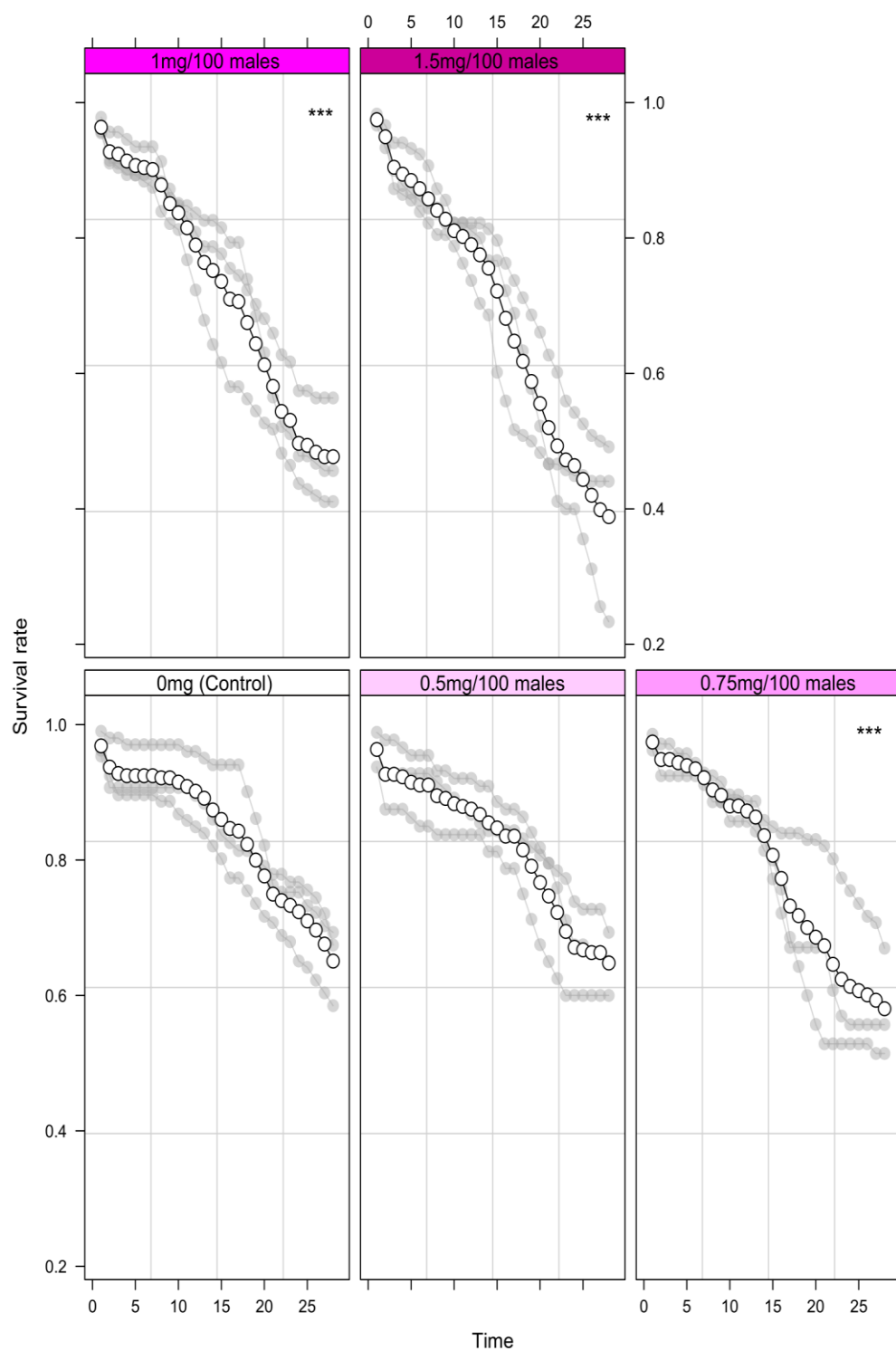
Results indicated that only the lowest dust amount (0.5 mg) did not significantly decrease longevity in male *Ae. aegypti* when compared to undusted controls ( $p = 0.888$ , Figure 3, Table S3) Interestingly, *Ae. albopictus* appeared less sensitive to dusting with the lowest two dust amounts (0.5 and 0.75 mg) having no significant impact on longevity in comparison to undusted controls ( $p = 0.951$  and  $0.166$  respectively, Figure 4, Table S4).

**Figure 3:**



Significant differences between experimental males (0.5, 0.75, 1 and 1.5 mg) and the control group (no dust) are indicated (\* $p < 0.05$ , \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). Individual values of the replicates are indicated in light grey and mean values as a solid line.

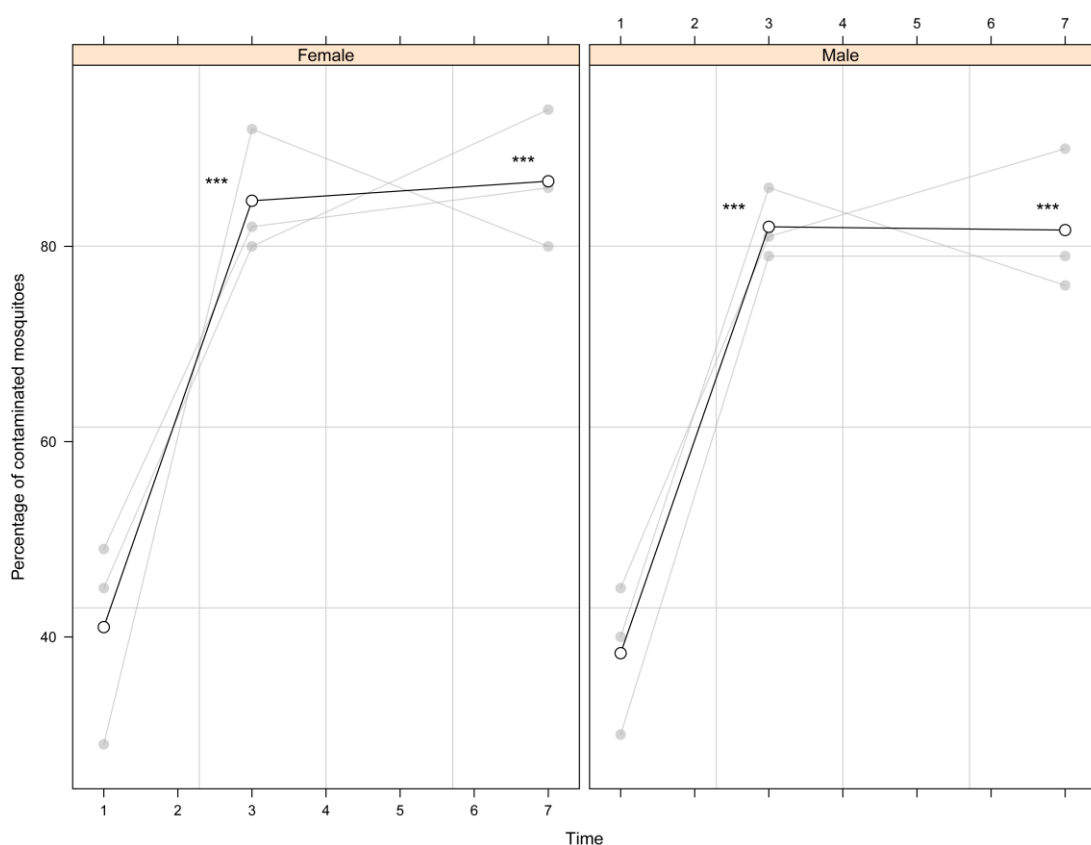
**Figure 4:**



Significant differences between experimental males (0.5, 0.75, 1 and 1.5 mg) and the control group (no dust) are indicated (\* $p < 0.05$ , \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). Individual values of the replicates are indicated in light grey and mean values as a solid line.

Visual inspection under a stereomicroscope was required to assess whether dust had been transferred to unmarked males and females as it was not noticeable either by the naked eye or under a UV light. There was no significant effect of sex on whether dust was transferred ( $p = 0.091$ ) but there were significantly more males and females displaying dust transfer on days 3 and 7 ( $p < 0.001$ ) in comparison to day 1 (Figure 5, Table S5).

**Figure 5:**



100 marked males (5 mg/100) were caged with 100 unmarked males and females. On days 1, 3 and 7 dusted males were removed from 3 cages and the remaining males and females screened for dust. Significant differences between the percentage of male and female mosquitoes contaminated with dust are indicated (\* $p < 0.05$ , \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). Individual values of the replicates are indicated in light grey and mean values as a solid line



## Discussion

The experimental work presented in this paper has allowed us to determine some key parameters relevant to developing a standardized small-scale method of marking *An. arabiensis*, *Aedes aegypti* and *Aedes albopictus* male mosquitoes prior to a release campaign such as that of a mark-release-recapture (MRR) study. We selected a dust colour, optimal amounts for all three species and verified that it did not impact mosquito longevity, nor was the mark easily transferred.

In addition to increasing immediate mortality, applying too much dust to an insect poses the problem that it cannot groom the excess off, thus decreasing mobility and interfering with their sensory organs. Thus, our first challenge was to determine the lowest possible amount of dust necessary to achieve a mark both visible to the naked eye and under UV light. Following this, we tested various colours with this dust amount to establish the most appropriate one. Different studies aimed at assessing the effect of different fluorescent dust colours on mosquito longevity have reported mixed results. Some studies indicate no difference in longevity between various dust colours [33] whereas others conclude that different colours exert varying effects upon mosquito longevity. One study noted blue as being particularly detrimental to mosquito longevity, even when various manufacturers of blue dust were tested. The authors also stated that blue dust is less visible after application in comparison to other colours [21]. No significant difference in longevity was noted after comparing the longevity between marked *male An. arabiensis* with different colours and unmarked controls. However, the blue and yellow mark was much less visible by eye and under UV light thus pink was chosen as the marking colour to be used for our marking

protocol. Furthermore, previous studies have also shown pink or red dusts to exert less of an impact upon longevity when compared to other colours [21].

After selecting our dust amount (5 mg/male) and colour (pink) for *An. arabiensis*, we verified that it did not negatively impact the longevity of marked males, which is consistent with what was stated by [21]. It is important to ensure that marked insects retain their mark for an appropriate amount of time. Once released, it will be necessary to identify sterile males and distinguish them from wild counterparts. We were able to successfully verify that our dust amount was sufficient to retain the visibility of the mark for upwards of one month, both with the naked eye and a UV light. We adopted to extrapolate our technique to *Ae. aegypti* and *Ae. albopictus*, however, due to their smaller body size, we concluded that a lower dust quantity would be required. The results of our study confirmed this initial postulation to be correct, with only the lowest dust weight of 0.5 mg found not to significantly decrease survival rate in *Ae. aegypti*. Our marking method proved successful when it was up scaled and used to mass-mark male *Ae. aegypti* prior to aerial release as part of a mark-release-recapture (MRR) study undertaken in Brazil in 2018 (unpublished data). The amount of dust used to coat 100 males (0.5 mg) was increased to 12 mg to mark batches of 2,400 males in 1 litre buckets and followed the same experimental guidelines used for small-scale marking experiments in the laboratory. In total, the technique was used to mark over 250,000 sterile males. Dickens and Brandt (2013) also found a significant decrease in survival in marked male *Ae. aegypti* in comparison to unmarked controls, although this result may not be surprising when considering they used 0.3 g of dust per 30 adult males [21]. Male *Ae. albopictus* were less sensitive to dusting however with mortality rates for 0.5 and 0.75 mg per 100 similar to undusted controls. Marini et al (2010) also

reported no significant impact upon survival when dusting male *Ae. albopictus* prior to release as part of a MRR study in Italy, although dust weight was not reported [34].

It is important that the mark is not easily transferable to the wild population of either males or females. The literature tends to suggest that fluorescent dust does not transfer between marked and unmarked mosquitoes when held together thus we conducted our own investigation to clarify or disprove this finding. A previous study conducted by [10] found that dust was not transferred when 30 marked and 30 unmarked *Culicoides* were confined for 24 hours within a trapping beaker. Fryer & Meek (1989) found that only 3% of unmarked *Psorophora columbiae* mosquitoes (9 out of 300) became marked during a 24 hour period of being caged with marked adults [35]. This result further confirmed reports from an earlier study which reported no dust transfer during the mating of *Drosophila pseudoobscura* or following heavy crowding of marked and unmarked individuals. However, marked insects were given time to groom themselves following dusting [36].

In stark contrast to the literature, our results indicated that marked males are indeed capable of transferring the mark to both males and females and in addition to this, the percentage of non-marked individuals that became marked increased over time. However, the dust transferred was not visible with the naked eye or under UV light. It was detectable only via examination with a stereomicroscope. We aimed to clarify the dust particle number transferred, which proved impossible as in most cases, the transferred dust was in a clump. Thus, even though we found a high percentage of unmarked males and females showing evidence of dust transfer it is unlikely to be relevant for sterile males marking wild males or females post-release. However, it is

an encouraging finding for techniques such as boosted SIT, which relies on the close contact of sterile males (coated with pyriproxyfen powder or densovirus) and wild females during mating and the transfer of powder [37, 38]. A marked male is very easily distinguishable from a male or female which has evidence of dust transfer. It is likely that when recollecting sterile males, a UV light will be used to detect their presence following recollections from traps and subsequent examinations within a laboratory setting. Dust transfer was not detectable in our study, unless using a stereomicroscope.

The short duration (24 hours) of holding marked and unmarked insects together may go some way to explain the lack of dust transfer in the above two studies. Or the close confinement of our marked and unmarked individuals in  $30 \times 30 \times 30$  cm bugdorm cages may explain why we saw such a high level of dust transfer. Alternatively, it may be the behaviour of this mosquito species itself that caused such a degree of dust transfer, as male *An. arabiensis* form swarms when mating [39]. It would be beneficial to repeat this experiment in large field cages to determine if indeed a limited spatial environment was responsible or by allowing the marked males sufficient time for grooming following dusting. However, it is likely that in a mass rearing facility, if immobilizing sterile males for marking, they will be packed into release canisters immediately afterwards, thus not allowing a window of time for grooming. If mosquitoes are self-marked or marked whilst active, it would then be possible to allow them time to groom prior to immobilizing them for packing.

The results of our study have demonstrated that immobilizing male mosquitoes prior to dusting does not significantly impact their survival. However, depending on the type

and scale of marking experiment being undertaken, immobilizing mosquitoes prior to marking may not always be feasible. Marking active male *Ae. albopictus* mosquitoes prior to a series of MRR experiments in Mauritius, has been shown to be effective [4]. Thus, our technique should be seen as another ‘tool’ in the SIT toolbox as opposed to the only or best available method.

There is much conflicting information in the literature regarding the use of fluorescent dust to mark mosquitoes. There does seem to be a general consensus that the dust colour and manufacturer can impact mosquito longevity negatively, in addition to the technique used to apply the dust. The main aim of our study was to provide a standardized guide to dust-marking several of the key disease-causing vectors of mosquito to deployed in small-scale releases of sterile male mosquitoes such as MRR studies. Our results highlight how determining the optimal dust quantity for one species, for example *An. arabiensis*, does not automatically mean that it can be inferred for another species (*Ae. aegypti*).

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## Supplementary Information

### Tables

**Table S1**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	0.8242	0.1385	5.951	2.67e-09
blue	-0.3336	0.2700	-1.235	0.217
yellow	-0.0704	0.2837	-0.248	0.804
pink	-0.2646	0.2744	-0.964	0.335

Fixed-effects coefficients of a mixed-effect binomial model of the impact of dust colour on survival in *Anopheles arabiensis*



**Table S2**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	0.7425	0.1367	5.433	5.56e-08
5 mg	0.0698	0.1923	0.363	0.717

Fixed-effects coefficients of a mixed-effect binomial model of the impact of pink dust (5mg/100) on survival in *Anopheles arabiensis*

**Table S3**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	-0.19665	0.17341	-1.134	0.2568
0.5 mg	0.02297	0.16374	0.140	0.8884
0.75 mg	-1.10257	0.21322	-5.171	2.33e-07
1 mg	-0.35453	0.16480	-2.151	0.0315
1.5 mg	-1.17341	0.18497	-6.344	2.24e-10

Fixed-effects coefficients of a mixed-effect binomial model of the impact of dust quantity on survival in *Aedes aegypti*

**Table S4**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	0.60744	0.13547	4.484	7.33e-06
0.5 mg	0.01104	0.18052	0.061	0.951
0.75 mg	-0.24543	0.17699	-1.387	0.166
1 mg	-0.71689	0.16886	-4.246	2.18e-05
1.5 mg	-1.01447	0.16680	-6.082	1.19e-09

Fixed-effects coefficients of a mixed-effect binomial model of the impact of dust quantity on survival in *Aedes albopictus*

**Table S5**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	-0.25087	0.13310	-1.885	0.0595
Sex	-0.18420	0.10912	-1.688	0.0914
Day	0.35680	0.02603	13.707	<2e-16

Fixed-effects coefficients of a mixed-effect binomial model of the occurrence of dust transfer between pink dusted (5 mg/100) male *Anopheles arabiensis* and undusted males and females after 1, 3 and 7 days

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## Chapter 7

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# Longevity of Mass-reared, Irradiated and Packed Male *Anopheles arabiensis* and *Aedes aegypti* Under Simulated Environmental Field Conditions

The results presented in this chapter have been published as the manuscript:

Culbert NJ, Maiga H, Bimbilé-Somda NS, Gilles, JRL, Bouyer, J, Mamai, W. Longevity of mass-reared, irradiated and packed male *Anopheles arabiensis* and *Aedes aegypti* under simulated environmental field conditions. Paras Vectors. 2018;11:603.

## Abstract

To ensure the success of a mosquito control programme that integrates the sterile insect technique (SIT), it is highly desirable to release sterile males with a maximal lifespan to increase release effectiveness. Understanding sterile male survival under field conditions is thus critical for determining the number of males to be released. Our study aimed to investigate the effect of mass rearing, irradiation, chilling, packing and release time on irradiated male mosquito longevity. *Anopheles arabiensis* and *Aedes aegypti* immature stages were mass-reared using a rack and tray system. Batches of 50 males irradiated at the pupal stage were immobilised, packed into canisters and chilled for 6 hours at 6 °C. Mosquitoes were then transferred either in the early morning or early evening into climate chambers set to simulate the weather conditions, typical of the beginning of the rainy season in Khartoum, Sudan and Juazeiro, Brazil for *An. arabiensis* and *Ae. aegypti*, respectively. The longevity of experimental males was assessed and compared to mass-reared control males subjected either to simulated field or laboratory conditions. The combined irradiation, chilling and packing treatments significantly reduced the longevity of both *An. arabiensis* and *Ae. aegypti* under simulated field conditions ( $P < 0.001$ ). However, packing alone did not significantly reduce longevity of *Ae. aegypti* ( $P = 0.38$ ) but did in *An. arabiensis* ( $P < 0.001$ ). Overall, the longevity of mass reared, irradiated and packed males was significantly reduced, with the median survival time (days) lower following an early morning introduction ( $4.62 \pm 0.20$ ) compared to an evening ( $7.34 \pm 0.35$ ) in *An. arabiensis* ( $P < 0.001$ ). However, there was no significant difference in longevity between morning ( $9.07 \pm 0.54$ ) and evening ( $7.76 \pm 0.50$ ) in *Ae. aegypti* ( $P = 0.14$ ). Our study showed that sterile mass-reared males have a reduced lifespan in comparison to laboratory-maintained controls under simulated field conditions, and that *An. arabiensis* appeared

to be more sensitive to the handling process and release time than *Ae. aegypti*. Longevity and release time are important parameters to be considered for a successful area-wide integrated vector control programme with a SIT component.

## Introduction

For several decades, the sterile insect technique (SIT) has been shown to be an efficacious and sustainable genetic approach with regard to the population management of several major pest insects, such as the New World screwworm *Cochliomyia hominivorax* (1), the tsetse fly *Glossina austensi* (2) and the Mediterranean fruit fly *Ceratitis capitata* (3), when deployed as part of an area-wide integrated pest management (AW-IPM) programme. Mosquitoes (Diptera: Culicidae) represent a serious threat worldwide for their vectorial capacity of major human disease pathogens. Several *Anopheles* and *Aedes* species are responsible for transmitting and spreading the most devastating disease pathogens including malaria, dengue, chikungunya, yellow fever, filariasis and the Zika virus. Over the last decade, substantial progress has been made regarding the development of the SIT package for mosquitoes including equipment and procedures (4). The chikungunya and the unprecedented Zika virus outbreaks in the Americas in 2015 have further reignited interest in using the SIT to control mosquitoes.

There are many potential stressors a sterile male mosquito must endure before it is finally released into the field, including mass-rearing, sex-separation, irradiation, marking, handling, immobilisation and packing. It is assumed that each element imposes a slight cost on the quality of the insect itself. The irradiation process has been attributed to reduced male mating competitiveness in insects (5). Thus, it is critical to determine the relative impact that each step has on insect quality to develop a standardised set of guidelines for each stage that imposes the least cost. However, there is little or no information regarding the post-pupal irradiation stages of mosquito SIT,

such as handling, transport and release. Recently, optimal transportation conditions for sterile male *An. arabiensis* adults have been studied (6).

The release of sterile male mosquitoes within the framework of a large-scale programme may involve releasing the insects aerially. To achieve this, sterile male mosquitoes would have to be packed, stored and transported in large numbers. Thus, it is of interest to investigate the impact packing has on sterile male mosquito longevity and additionally the maximum density. Mosquitoes are produced in the laboratory under stable and favourable environmental conditions; however, they will be released into the field where environmental conditions undergo daily and seasonal variation. Thus, it is of concern how long these mass-reared sterile males will survive under challenging field conditions when released. Furthermore, it may be useful to determine if the time of day the release occurs has an impact upon the quality of the insect. Environmental conditions such as temperature and relative humidity (RH) can fluctuate drastically throughout the day; thus, preferred conditions need to be determined. Aerial releases will be most effective when carried out when the conditions are favourable, to optimise the insect's survival and post-release performance. For example, aerial releases of sterile fruit flies are typically carried out early in the day; in Reynosa and Tijuana, both situated on the Mexican border, releases are carried out mid- and late morning, as is the case in the Los Angeles basin (7)

The work presented here aimed to estimate the survival of male mosquitoes when exposed to simulated field conditions and to determine the effect, if any, the process of packing has on sterile male longevity whilst undergoing chilling. Additionally, we investigated whether releasing irradiated males in the early morning or early evening

was better by simulating natural environmental conditions for both *An. arabiensis* and *Ae. aegypti*. Lastly, we compared the longevity of irradiated males against unirradiated, mass-reared males that did not undergo chilling or packing but were exposed to the simulated environmental conditions of Khartoum and Juazeiro or standard laboratory rearing conditions.

## **Materials and Methods**

### **Source of mosquito colonies and mass rearing procedures**

All experiments were performed using two established mosquito colonies, *An. arabiensis* (Dongola strain) and *Ae. aegypti* (Brazil strain), originating from the Northern State of Sudan (since 2005) and Juazeiro, Brazil (since 2012), respectively. Neither colony has been regenerated since the colonisation dates detailed above. They were maintained at the Insect Pest Control Laboratory (IPCL) of the joint Food and Agricultural Organisation/International Atomic Energy Agency (FAO/IAEA) Division of Nuclear Techniques and Agriculture, Seibersdorf, Austria, under controlled temperature, RH and light regimes ( $27 \pm 1$  °C,  $70 \pm 10\%$  RH, 12:12 h light:dark (L:D) photoperiod with 1 h periods of simulated dawn and dusk). Eggs used for these experiments were generated following the *An. arabiensis* and *Ae. aegypti* mass-rearing procedures developed at the IPCL (8-9). Larvae were mass-reared in a large climate-controlled room (with an area of 88 m<sup>2</sup>) where temperature and humidity were maintained at  $30 \pm 1$  °C,  $70 \pm 10\%$  RH, respectively.

To mass rear *An. arabiensis*, 4 l of deionised water was added to each of the 50 larval mass rearing trays and placed within a mechanized stainless-steel rack developed at the IPCL (10). Water was added 1 day before the addition of eggs to allow the water

temperature to acclimatise to the ambient air temperature. Following the egg quantification method described in Maiga et al. (11), 4000 eggs were then added to each mass rearing tray, within a plastic ring floating on the water surface. Larvae were fed daily with 1% (wt/vol) IAEA diet developed and described at IAEA (12), using the feeding regime described in Soma et al. (13). *Aedes aegypti* larvae were reared within mass rearing trays, with a larval density of approximately 18,000 first-instar larvae (L<sub>1</sub>) per tray containing 5 l of deionized water and fed with 7.5% IAEA diet (50 ml on day 1, 100 ml on day 2, 150 ml on day 3, 200 ml on day 4 and 50 ml from day 5 onwards) (14).

### **Pupae collection and irradiation**

Twenty-four hours after *An. arabiensis* pupae were first observed, the rack was tilted, and pupae separated from larvae by placing them in an Erlenmeyer flask containing dechlorinated water and swirling (15). Male pupae were separated from females under a stereomicroscope by distinguishing differences in genitalia (15). *Aedes aegypti* pupae were sexed mechanically by using a Fay-Morlan (16) glass sorter as redesigned by Focks (John W. Hock Co., Gainesville, FL, USA (17) prior to further examination under a stereomicroscope, ensuring pure batches of males. To be consistent with ongoing field pilot trials by Member States, irradiation was carried out at the pupal stage. Twenty-four to 26-hour-old *An. arabiensis* pupae were exposed to 75 Gy, and 44–48-hour-old *Ae. aegypti* pupae were irradiated at 70 Gy in a self-contained <sup>60</sup>Co Gamma Cell 220. Male pupae were irradiated in batches of 200 without water. The actual doses of irradiated pupae were quantified using Gafchromic MD film (International Specialty Products, NJ, USA). The actual doses received for *An. arabiensis* and *Ae. aegypti* were  $86.5 \pm 1$  Gy and  $77.5 \pm 2$  Gy, respectively.



### **Setting up environmental field conditions inside climate chambers**

Khartoum, Sudan and Juazeiro, Brazil environmental conditions were selected for the presence of these species and SIT pilot trials for *An. arabiensis* and *Ae. aegypti*, respectively. For *An. arabiensis*, the onset rainy season period was selected due to the fact that during the dry season, mosquito densities drop dramatically, and the mosquito population builds up gradually from the first rains toward the rainy season and in the northern part of Sudan, the seasonal larval population follows the rise and fall of the Nile River level (18). We assumed therefore that this transition period (early rainy season) could be the best period to start mosquito releases because the target mosquito population is already low and so that high ratios of sterile to wild insects would be easily obtained. A climate chamber (Sanyo MLR 315H, Osaka, Japan) was programmed to provide the temperature and RH on a typical April 17th, based on data obtained from a weather station at Khartoum International airport, Sudan, and averaged over 5 years. Twelve-step cycles were designed to reproduce the natural climatic variations monitored in the field. Experiments were conducted with a photoperiod of 12L:12D. The above process was repeated for *Ae. aegypti* with conditions set to simulate those of Juazeiro, Brazil, based on yearly hourly averages over 3 years. Data loggers (Onset Hobo data loggers, Bourne, MA, USA) were placed inside the chambers to monitor the temperature and humidity throughout the experiment. The actual data (averaged hourly records), presented in Fig. 1, simulated as closely as possible field data for Khartoum (Fig. 1a) and Juazeiro (Fig. 1b). Another chamber was set to 6 °C, 50% RH for the chilling process.

### **Effect of packing on sterile male longevity**

After the irradiation of pupae, sterile adults should be packed for transportation to the release area. Following irradiation, pupae were separated into batches of approximately 60 pupae (three replicates) and left to emerge in small Bugdorm cages (BugDorm, Taipei, Taiwan; 15 × 15 × 15 cm) with access to 5% and 10% sucrose solution for *An. arabiensis* and *Ae. aegypti*, respectively. On day 3 post-emergence, 3 cages containing 50 irradiated males were chilled at 4 °C for 5–10 min to immobilise the adults. After immobilisation, they were packed into plastic tubes (D × H: 1.5 × 4 cm) with an open end covered by a small square of mesh to allow ventilation and secured with an elastic band. Control males remained in their original Bugdorms and were not subject to packing. Both the experimental and control adults were placed inside a climate chamber set at 6 °C, 50% RH for a period of 6 h. After chilling, all cages were returned to laboratory conditions (27 ± 1 °C, 70 ± 10% RH) with experimental males removed from the packing tubes and returned to their original Bugdorm cages. Mortality checks were carried out daily in both control and experimental cages until no living adults remained.

#### **Assessing longevity of irradiated males under simulated field conditions and preferred time of day to release**

Three batches of 50 sterile males, packed and chilled for 6 h were placed in the climate chamber at 6:00 h (treatment 1: morning) at the same time as the males which were not subjected to irradiation, packing or chilling (control 1: field conditions). Additionally, three batches of 50 control males which were not exposed to irradiation, packing or chilling were maintained at laboratory conditions (27 ± 1 °C, 70 ± 10% RH) (control 2: lab conditions). Three further batches of 50 sterile males were packed and chilled at 6 °C, 50 % RH for 6 h and then exposed to field conditions at 18:00 h

(treatment 2: evening). Mortality was recorded daily until no adults remained. Unirradiated controls for the first 2 experiments were maintained under standard laboratory conditions.

### **Statistical analysis**

Graphics were produced and all statistical analyses were performed using Microsoft Excel 2013 (Microsoft®, Redmond, WA, USA) and GraphPad Prism v.5.0 (Windows, Graphpad Software, La Jolla California, USA; [www.graphpad.com](http://www.graphpad.com)). The longevity of mosquitoes under various experimental conditions was analysed using Kaplan-Meier survival analyses. The log-rank (Mantel-Cox) test was used to compare the level of survival between different treatments and controls. To counteract the problem of multiple comparisons the Bonferroni correction method was applied for each pair of groups.

## **Results**

### **Effect of packing on male longevity of *Anopheles arabiensis* and *Aedes aegypti***

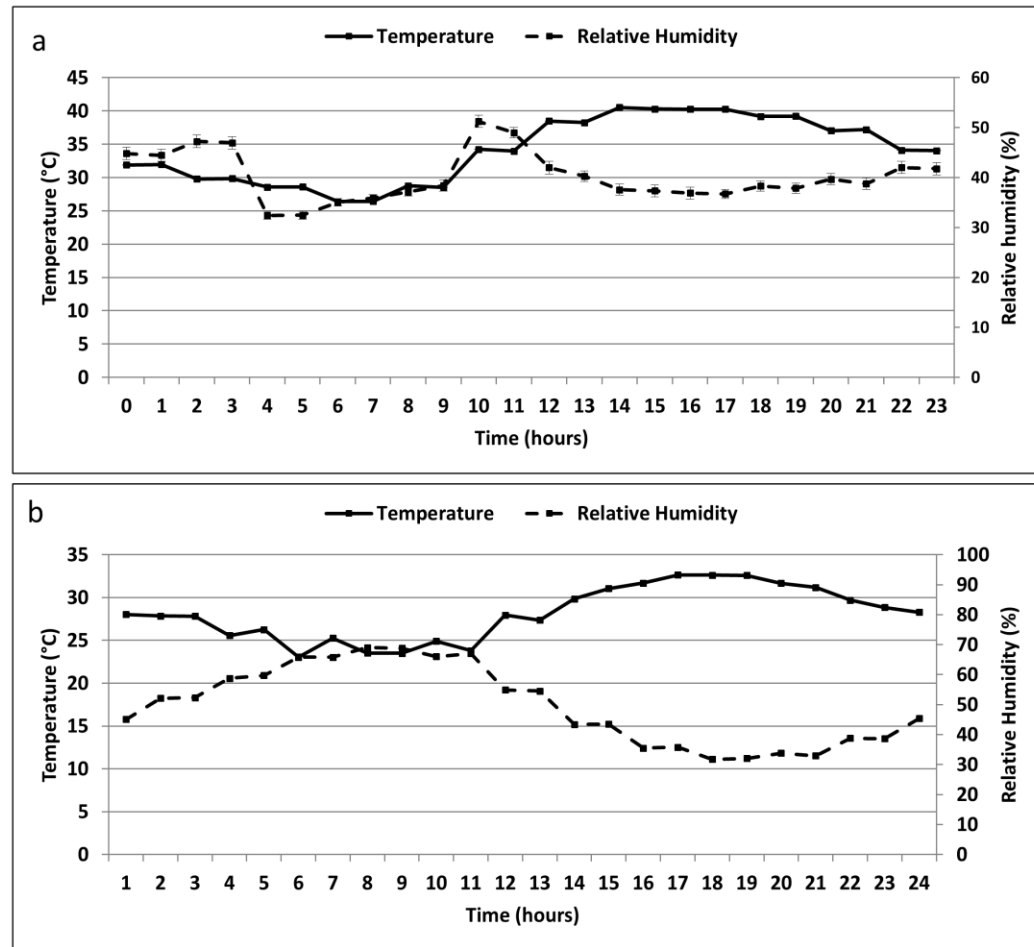
The longevity of 50 sterile males packed in a small tube and chilled at 6 °C for 6 h was compared to 50 sterile males chilled at 6 °C for 6 h in a small Bugdorm cage. The longevity was followed in laboratory conditions and the survival curve is presented in Fig. 2. Statistical tests between all treatments were summarized in Table 1. The analyses showed that the packing treatment significantly reduced the longevity of *An. arabiensis* males (Fig. 2a, Table 1, Log-rank (Mantel-Cox) test  $\chi^2 = 18.15$ ,  $df = 1$ ,  $P < 0.001$ ) and did not affect that of *Ae. aegypti* males (Fig. 2b, Table 1, Log-rank (Mantel-Cox) test  $\chi^2 = 0.76$ ,  $df = 1$ ,  $P = 0.38$ ).

**Table 1:**

	Treatments for comparison	$\chi^2$	<i>df</i>	<i>P</i>
<i>An. arabiensis</i>	Packed × unpacked	18.15	1	< 0.001
	Treatment 1 (Morning) × Treatment 2 (Evening)	41.09	1	< 0.001
	Treatment 1 (Morning) × Control 1 (field conditions)	80.45	1	< 0.001
	Treatment 1 (Morning) × Control 2 (lab conditions)	331.00	1	< 0.001
	Treatment 2 (Evening) × Control 1 (field conditions)	15.60	1	< 0.001
	Treatment 2 (Evening) × Control 2 (lab conditions)	91.45	1	< 0.001
	Control 1 (field conditions) × Control 2 (lab conditions)	274.30	1	< 0.001
<i>Ae. aegypti</i>	Packed × unpacked	0.76	1	0.38
	Treatment 1 (Morning) × Treatment 2 (Evening)	2.21	1	0.14
	Treatment 1 (Morning) × Control 1 (field conditions)	149.70	1	< 0.001
	Treatment 1 (Morning) × Control 2 (lab conditions)	363.60	1	< 0.001
	Treatment 2 (Evening) × Control 1 (field conditions)	176.20	1	< 0.001
	Treatment 2 (Evening) × Control 2 (lab conditions)	409.70	1	< 0.001
	Control 1 (field conditions) × Control 2 (lab conditions)	124.00	1	< 0.001

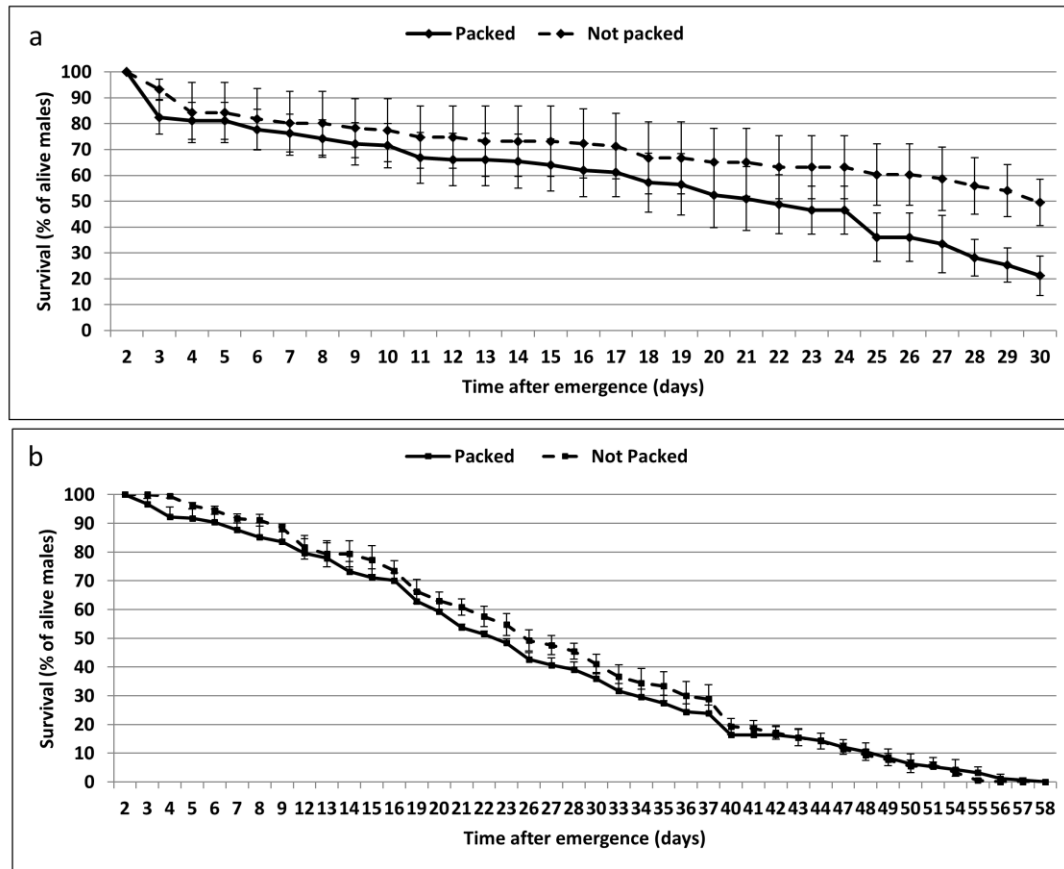
Results of log-rank (Mantel-cox) test analysis for the effect of packing, environmental treatments and preferred time of day to release on the longevity of *Anopheles arabiensis* and *Aedes aegypti* males.

**Figure 1:**



Mean ( $\pm$  standard error, SE) daily environmental conditions of temperature (solid line) and relative humidity (dashed line) recorded in the climate-controlled chambers simulating the natural conditions in Khartoum (Sudan) for *An. arabiensis* (a) and Juazeiro (Brazil) for *Ae. aegypti* (b)

**Figure 2:**



Mean ( $\pm$  standard error, SE) longevity of male *Anopheles arabiensis* (a) and male *Aedes aegypti* (b) recorded under packed (solid line) and unpacked (dashed line) conditions.

### Longevity of *An. arabiensis* and *Ae. aegypti* males under different environmental treatments and time of day to release

When exposed to simulated field conditions, the combination of irradiation, chilling and packing (treatment 1 vs control 1 and treatment 2 vs control 1) significantly reduced the longevity for *An. arabiensis* (Fig. 3a, Table 1, Log-rank (Mantel-Cox) test  $\chi^2 = 80.45$ ,  $df = 1$ ,  $P < 0.001$  and  $\chi^2 = 15.60$ ,  $df = 1$ ,  $P < 0.001$  for treatment 1 vs control 1 and treatment 2 vs control 1, respectively) and *Ae. aegypti* (Fig. 3b, Table 1, Log-rank (Mantel-Cox) test

$\chi^2 = 149.7$ ,  $df = 1$ ,  $P < 0.001$  and  $\chi^2 = 176.2$ ,  $df = 1$ ,  $P < 0.001$  for treatment 1 vs control 1 and treatment 2 vs control 1). In addition, the combination of irradiation, chilling, packing and laboratory conditions (treatment 1 vs control 2) significantly reduced male longevity for *An. arabiensis* (Fig. 3a,  $\chi^2 = 331.0$ ,  $df = 1$ ,  $P < 0.001$ ) and *Ae. aegypti* (Fig. 3b,  $\chi^2 = 363.6$ ,  $df = 1$ ,  $P < 0.001$ ).

The exposure of male mosquitoes to simulated field conditions (control 1) significantly reduced longevity compared to those maintained under laboratory conditions (control 2) for *An. arabiensis* (Fig. 3,  $\chi^2 = 274.3$ ,  $df = 1$ ,  $P < 0.001$ ) and *Ae. aegypti* (Fig. 3b,  $\chi^2 = 124.0$ ,  $df = 1$ ,  $P < 0.001$ ). For *Ae. aegypti*, there was not significant effect on longevity (Fig. 3b,  $\chi^2 = 2.209$ ,  $df = 1$ ,  $P = 0.1372$ ) for males introduced inside the climatic chamber (field conditions) in the morning (6:00 h) or in the evening (18:00 h). The median survival time was  $9.07 \pm 0.54$  and  $7.76 \pm 0.50$  days for morning and evening, respectively (Table 2). Conversely, for *An. arabiensis*, males introduced inside the climatic chamber in the evening (18:00 h) had a higher longevity than those introduced in the morning (6:00 h) (Fig 3a, Log-rank (Mantel-Cox) test  $\chi^2 = 41.09$ ,  $df = 1$ ,  $P < 0.001$ ). The median survival time was  $4.62 \pm 0.20$  days for the morning and  $7.34 \pm 0.35$  days for the evening (Table 2).

**Table 2:**

Species	Treatment 1 (morning) <sup>a</sup>	Treatment 2 (evening) <sup>b</sup>	Control 1 (field conditions) <sup>c</sup>	Control 2 (lab conditions) <sup>d</sup>
<i>An. arabiensis</i>	4.62 ± 0.20	7.34 ± 0.35	8.33 ± 0.42	19.09 ± 1.07
<i>Ae. aegypti</i>	9.07 ± 0.54	7.76 ± 0.50	21.28 ± 0.56	39.94 ± 0.98

Mean (± SE) longevity (days) of *Anopheles arabiensis* and *Aedes aegypti* males exposed to different environmental treatments:

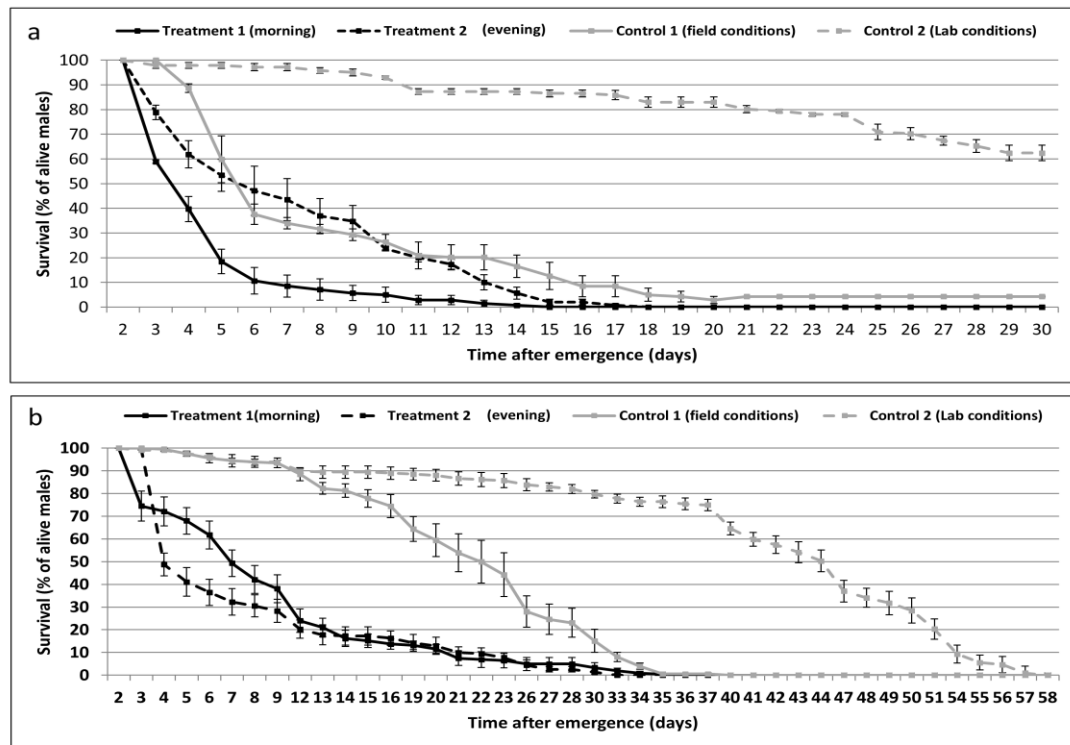
<sup>a</sup>Treatment 1: mass-rearing + irradiation + packing + field conditions + introduced in the climate chamber at 6:00 h

<sup>b</sup>Treatment 2: mass-rearing + irradiation + packing + field conditions + introduced inside the climatic chamber at 18:00 h

<sup>c</sup>Control 1: mass-rearing + field conditions + introduced inside the climatic chamber at 6:00 h

<sup>d</sup>Control 2: mass-rearing + laboratory conditions + introduced at the lab at 6:00 h

**Figure 3:**





Mean ( $\pm$  standard error, SE) longevity of male *Anopheles arabiensis* (a) and male *Aedes aegypti* (b) under different treatments. Treatment 1: mass-rearing + irradiation + packing + field conditions + introduced in the climate chamber at 6:00 h. Treatment 2: mass-rearing + irradiation + packing + field conditions + introduced inside the climatic chamber at 18:00 h. Control 1: mass-rearing + field conditions + introduced inside the climatic chamber at 6:00 h. Control 2: mass-rearing + laboratory conditions + introduced in the lab at 6:00 h.

## Discussion

Understanding sterile male longevity is of utmost importance for the effective implementation of SIT technology. Our research aimed to investigate the longevity of sterile male mosquitoes when exposed to simulated field conditions. Additionally, we aimed to determine whether there is an effect of the process of packing on sterile male mosquito longevity. The impact of packing sterile males, such as would be performed prior to transporting adults from a rearing facility to a release site was explored. Additionally, we simulated environmental conditions for morning and evening releases for both *An. arabiensis* and *Ae. aegypti* to determine whether time of day had any impact upon subsequent longevity.

Packing was found to significantly decrease the longevity of *An. arabiensis*. This result is inconsistent with what was noted when comparing the longevity of compacted vs non-compacted *An. arabiensis* in an earlier publication, where no significant decrease was observed (6). The different methodology between the experiments within this study and that of our earlier publication may indeed have contributed to the different results. For example, in our previous study, male *An. arabiensis* were not subject to irradiation. Perhaps this is why packing did not significantly decrease survival between packed and unpacked experimental males, but in comparison to control males, both experimental

treatment groups (packed and non-packed) did display a significantly lower longevity (6). In the packing experiment detailed within this manuscript, males were subject to irradiation. Therefore, it may be a synergetic effect of irradiation, packing and chilling which caused a significant decrease in longevity for *An. arabiensis* in our study. This synergetic effect has been shown in other species used within programmes with a sterile insect component. Sterile male fruit flies (*Ceratitis capitata*) were observed to exhibit significantly reduced flight ability and mating competitiveness when chilled in crowded conditions. However, independently, chilling or crowding did not cause significant decreases in either parameter (6, 19) Interestingly, the same result was not observed for *Ae. aegypti*. *Aedes aegypti* and *Ae. albopictus* mosquitoes appear to be less susceptible to chilling and compaction as compared to *Anopheles* (Culbert, unpublished data). We suspect this divergent response between species may reflect their different levels of tolerance to stressors. However, it cannot be ruled out that other factors, such as long-term colonisation in *An. arabiensis* (13 years, without regeneration), might be involved in causing the fragility observed during packing.

*Anopheles arabiensis* exposed to simulated field conditions when released in the evening had a significantly higher survival rate compared to those released at early morning. In the evening, conditions were much warmer (around 39 °C) than that in the morning (26 °C). However, temperatures increase in the morning (from 26 °C to 45.5 °C) while in the evening temperatures decrease (from 39 °C to 34 °C). *Anopheles arabiensis* exposed to high and decreasing temperatures seem to adapt much better than those exposed to low and increasing temperatures. It has been demonstrated that a brief exposure to extreme heat or cold often elicits physiological responses such as heat shock proteins that improve

an organism's thermal tolerance (20). *Anopheles arabiensis* is well known to favour hot, dry conditions in the wild (21), most notably in Sudan (22), the origin of our laboratory strain. This may have contributed to the higher survival observed in males which underwent a simulated evening release but conflicts with the literature, which states insects generally lose their thermal tolerance upon domestication (23). There is considerable variation regarding *Aedes* survival in the field, due to the limited temperature ranges at which field studies are conducted in addition to the relatively small sample sizes used in mark release recapture (MRR) studies (24). In *Ae. aegypti*, we found no significant difference in longevity between an early morning and an evening release. This may be because the shift in temperature between morning and evening was not as great as that which *An. arabiensis* were subjected to, with the fluctuation range closer to their normal rearing conditions within the laboratory.

Sterile male insects have one purpose, to mate with wild females and thus induce sterility within the target population. It is critical that sterile insects survive as long as possible in the field to ensure the success of a SIT programme. If sterile males are of poor quality and exhibit a reduced longevity, the frequency of releases coupled with the number of insects required for each release will have to be increased in order to preserve the overflooding ratio (25), which will increase costs. Mass-rearing, irradiation, handling and release methods can all contribute to a reduced lifespan in sterile insects. Often, longevity studies are conducted within the laboratory and may not be an accurate reflection of actual field survival; for example, due to the controlled climatic conditions and the absence of predation. This may explain why our results, when conducted to more accurately reflect a field setting, differ from what has previously been observed within our laboratory studies.

*Anopheles* males have been shown to survive on average for 20 days within a laboratory setting, whilst wild types display a much shorter lifespan averaging only 5–10 days (26). In *Aedes* species, wild male longevity is less documented but does seem to be dependent on season, with *Ae. aegypti* populations in Vietnam found to exhibit a much higher survival in either cool or hot dry seasons when compared to the cool and wet season (27). The results of this study highlight the fact that each step before release, such as the mass-rearing process, irradiation, handling and transport, can cause a cumulative detrimental effect on the longevity of sterile mosquitoes and perhaps their overall quality. This is further emphasised when conditions are set to simulate field conditions, as opposed to a controlled laboratory setting. It would be of interest to conduct quality control tests, such as investigating flight ability and or mating competitiveness experiments, to ascertain if these parameters are impaired too. Understanding which treatments impact sterile male quality most and rectifying those parameters will ultimately lead to a higher quality of insect and a more successful SIT programme.

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## Chapter 8

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# Field performance of a mosquito-releasing drone in Brazil

The results presented in this chapter have been submitted as a manuscript to Science Robotics on December 18<sup>th</sup> 2019. The manuscript was accepted subject to minor modifications on March 23<sup>rd</sup> 2020.

## **Abstract**

Genetic control of mosquito vectors of malaria, dengue, yellow fever and Zika, is rapidly expanding due to the limitations of other techniques such as the use of insecticides. For the Sterile Insect Technique, it is crucial to release sterile mosquitoes by air to ensure homogeneous coverage, especially in large areas. A fully automated adult mosquito release system operated from an unmanned aerial vehicle was developed and tested in Brazil, which allowed a homogeneous dispersal of sterile male *Aedes aegypti*, while maintaining their quality. This will greatly facilitate the implementation of genetic control methods for mosquito vectors.

## **Main Text**

According to the World Health Organization (WHO), vector-borne diseases account for 17% of infectious diseases leading to more than one million human casualties each year. This includes in order of importance, malaria, lymphatic filariasis and arboviruses like dengue, yellow fever and Zika. In a growing number of countries, awareness of the toxicity of insecticides to living organisms and ecosystems is resulting in governments banning more and more of these chemicals. Moreover, resistance to pyrethroids, the most commonly used group of insecticides against insects, is increasing, which could in the short-term result in their complete ban. Therefore, the WHO's global vector control response 2017-2030 urgently demands for alternative mosquito control tactics, particularly against *Aedes* vectors (1).

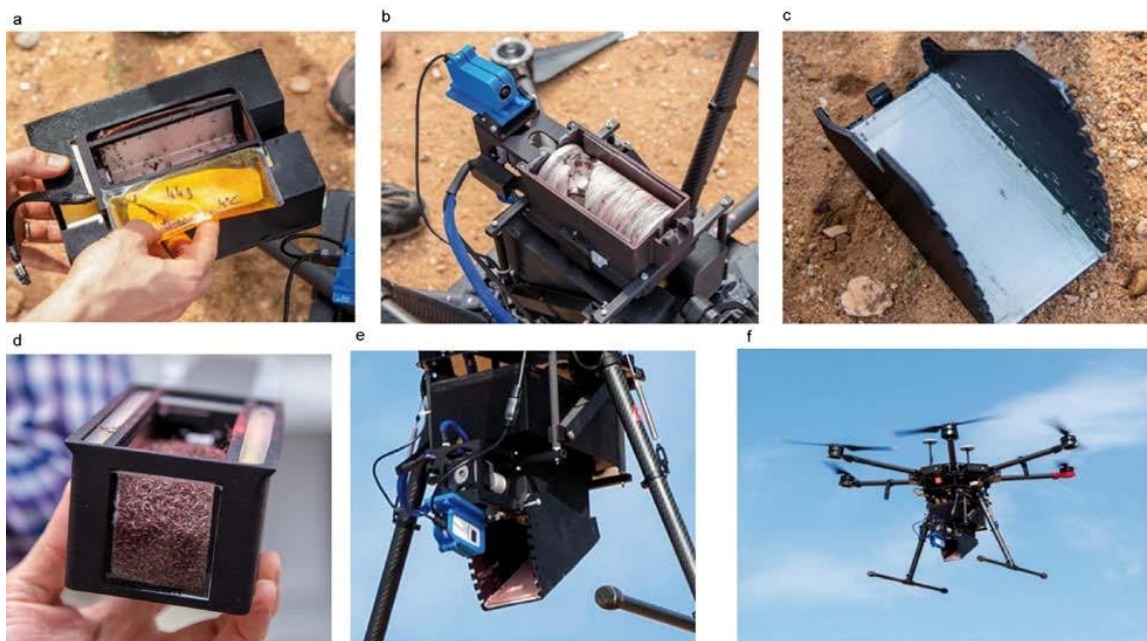


Many new mosquito control methods have become available (2) and some genetic control tactics show great promise (3). The sterile insect technique (SIT) is the ancestor genetic control method and has been used with great success against insect pests of agriculture and livestock, i.e. the New World screwworm (4), fruit flies (5), moths (6) and tsetse flies (7, 8). Very recent genetic control tactics include (1) the use of symbionts like *Wolbachia* for the incompatible insect technique (IIT) that was successfully combined with the SIT for the suppression of *Aedes albopictus* (9) or for population transformation using virus blocking strains (10-12), (2) genome editing (13) and, (3) the use of transgenic insect strains (10, 14). Gene drive is the latest developed technology (15, 16) and associated with male determining factors (17), now described in both *Anopheles* (18) and *Aedes* (19) families, it is expected to become a powerful mosquito control tool but it is not ready to be released in the field yet (20).

Aerial release approaches will be required to ensure cost-effective releases of the sterile male mosquitoes, especially when large areas need to be covered. A fully automatic release system was developed for the release of adult sterile male *Aedes* mosquitoes that can be operated from an unmanned aerial vehicle (UAV), commonly known as a drone. Mosquitoes have long fragile legs and delicate wings which makes the design of a release system that does not cause injuries and hence, reduce their quality, very challenging. From an entomological perspective, the main challenges to address were compaction, chilling and the development of a conveyor system, to permit stacking an adequate number of mosquitoes per flight, ensuring their complete immobilization and controlling the release flow rate without causing injuries (21). From a mechanical engineering perspective, the release platform included the release mechanism that consisted of an insulated storage unit, a

mechanism that ejects the mosquitoes onto a release area ramp, and onboard electronics featuring sensors and cameras to control and monitor the state of the mechanism and mosquitoes (Figure 1). A custom-made Android-based software application was in addition developed to operate mosquito release flights autonomously which would make the planning and implementation of the releases more effective (Suppl. Online Information).

**Figure 1:**



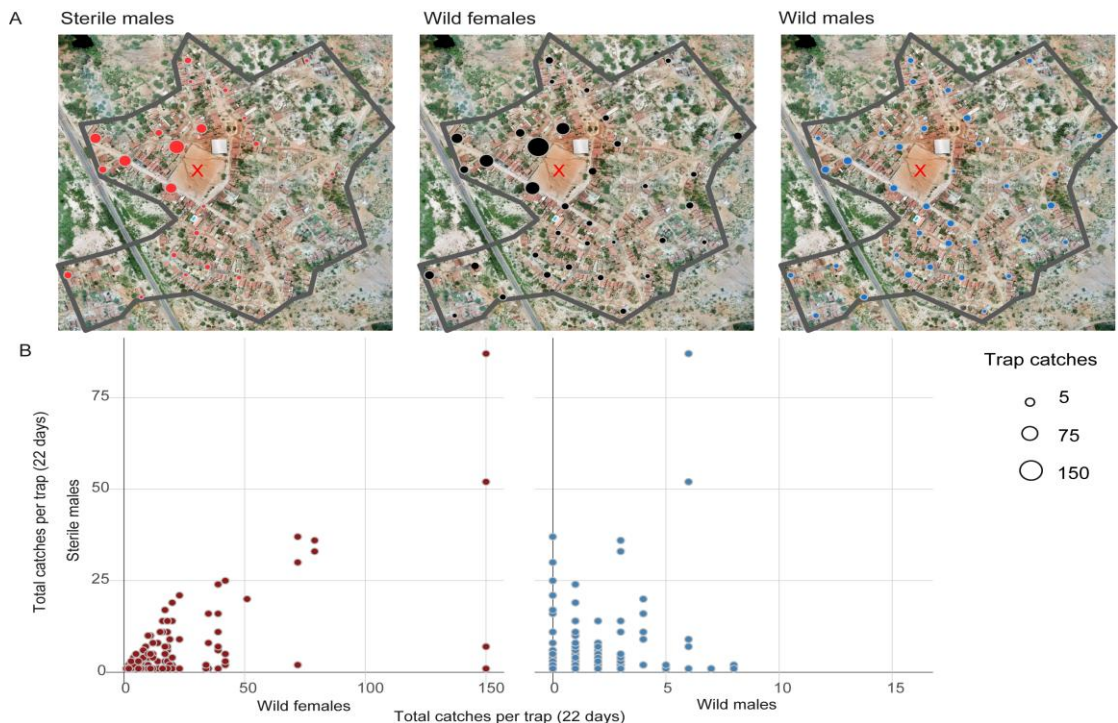
The adult mosquito release system operated from an unmanned aerial vehicle (UAV). A: an empty canister with insulated walls after a release flight. Note the yellow phase-change material (PCM) pack in half-frozen state, B: the conveyor system with the release monitoring camera (blue) shown upside-down, C: the release ramp, D: a canister filled with 50,000 marked mosquitoes, E: the release mechanism in flight, and F: a fully-assembled aerial mosquito release system attached to a DJI M600 UAV in flight.

In the laboratory, the effect of the different treatments (compaction, chilling, release) on the quality of the released mosquitoes was assessed using a standardized flight ability test that determines the proportion of adult mosquitoes escaping from a 25 cm tall vertical tube ((21),

Suppl. Online Information). The percentage of flyers was significantly correlated with the proportion of mosquitoes with damaged wings and legs ( $r = -0.98$ ,  $p = 0.02$ , Suppl. Online Information). Male *Aedes aegypti* mosquitoes proved to be very sensitive to compaction up to  $1.2 \text{ g/cm}^2$  and therefore, a release cassette was developed with a maximal depth of 5 cm that contained no more than 50,000 *Ae. aegypti* males (Suppl. Online Information). Moreover, at temperatures below  $8^\circ\text{C}$  the mosquitoes were behaving like inert particles and their quality was reduced (21) whereas at  $11^\circ\text{C}$ , some mobility was restored. Therefore, an insulated container was developed that could hold phase-change material packs (S8, PureTemp®, MN) to maintain the temperature between 8 and  $10^\circ\text{C}$  throughout the flight. Mosquitoes exposed to these temperatures for 1 to 4 hours, became active again after 40 to 60 s when transferred to ambient temperatures. In view that *Ae. aegypti* males have an estimated average free fall speed of  $2.5 \text{ m / sec}$ , 50 and 100 m were selected as potentially appropriate release altitudes (Suppl. Online Information). Finally, two conveyor systems were compared, i.e. a conveyor system commonly used to release fruit flies (22) and a cylinder system initially developed to release tsetse flies (23) (Figure 1). The tsetse fly cylinder system, which is smaller and lighter than the fruit fly conveyer system, resulted in better-quality mosquitoes (higher flight rate,  $p=0.02$ , Suppl. Online Information). The competitiveness of irradiated sterile males that had been exposed to the release mechanism of the final design was assessed in larger cages ( $60 \times 60 \times 60 \text{ cm}$ ) under experimental laboratory conditions, and proved to be similar to that of untreated control mosquitoes, i.e. a Fried index (24) of 0.66 (SD 0.06) ( $t = -0.036467$ ,  $df = 3.9977$ ,  $p = 0.97$ ). To simulate the forces experienced by the mosquitoes when released from the drone, a wind tunnel experiment confirmed that a wind speed of 7 to 19 meters / s (25-68 km/h) did not reduce the quality of the males released with this system ( $p>0.09$ , Table S2).

In March 2018, the UAV platform was tested in a field trial in Carnaíba do Sertão, Juazeiro, Brazil, (Figure 2 & movie S1). A total of 50,400 sterile irradiated males were either released from a central point on the ground or released from an UAV in stationary flight at an altitude of 50 or 100 m (Table 1). The mosquitoes were recaptured with 35 baited BG monitoring™ traps (Biogents, Germany) in the 20 ha trial area. More ground-released mosquitoes (1.60%, SD 0.42%) were recaptured than UAV-released mosquitoes ( $p < 10^{-3}$ ), and recapture rate of mosquitoes released from an altitude of 50 m (0.27%, SD 0.01%) was significantly better than those released from an altitude of 100 m (0.07%, SD 0.02%,  $p < 10^{-3}$ ). Survival of the three groups was similar ( $p > 0.46$ , stats, Suppl. Online Information) but their average dispersal increased with release altitude ( $p = 0.011$ ), i.e. from 83 m (SD 21 m) to 133 m (SD 22m) and 153 m (SD 7m) for mosquitoes released from the ground, from 50 and 100 m, respectively.

**Figure 2:**

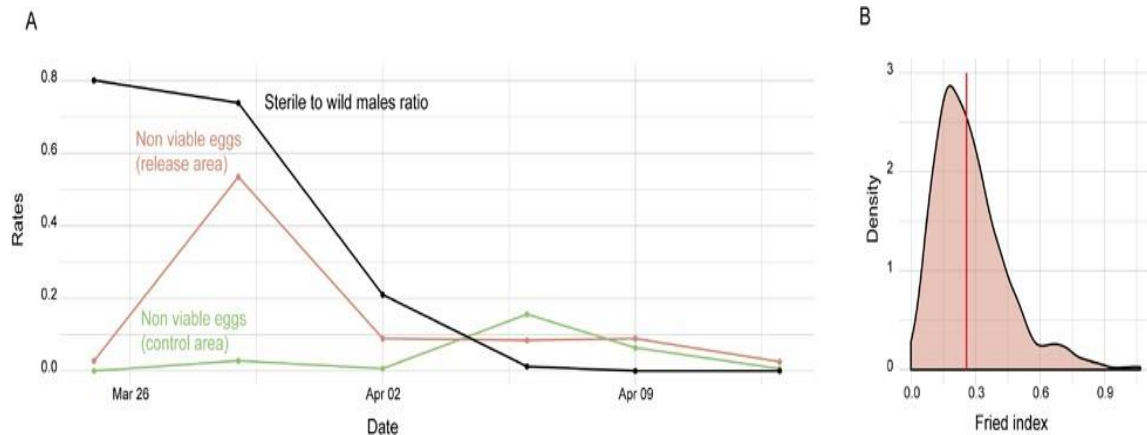


Results of a mark-release-recapture experiment in Carnaíba do Sertão, Brazil. (A) Map of the total catches of *Aedes aegypti* with BG monitoring <sup>TM</sup> traps (Biogents, Germany) deployed from 20<sup>th</sup> March to 11<sup>th</sup> April 2018. Each data point represents the total catch of one trap during the experimental period. The red cross represents the location of point releases in the middle of a football field. (B) Relationship between sterile males catches and those of wild females and males. Catches of sterile males were significantly correlated with those of wild females (cor = 0.62, t = 3.3528, df = 18, p-value = 0.0035) and wild males (cor = 0.53, t = 2.6677, df = 18, p-value = 0.016).

To implement this trial, the flight speed of the drone was set at 10 m/s and the cylinder speed at 2 revolutions per minute which allowed a release rate of ~5000 sterile males per ha. Approximately 12 minutes were needed to cover 20 ha. During the flight, the temperature exceeded 10°C but remained below 18°C (Fig S. 10). A total of 165,400 sterile males were released along release lines separated with a swath of 80 m and marked mosquitoes were recaptured in 21 out of the 35 monitoring traps (63%, fig.1), which indicated a uniform release pattern. The recapture rate of 0.32% (SD 0.09%) in this study was better than that of RIDL® (Release of Insects carrying a Dominant Lethal) *Ae. aegypti* males in Brazil (0.04%) (14) or that of *Ae. albopictus* males used in an IIT-SIT trial in China (0.09%, SD 0.07%), both released from the ground (9). The significant correlation between sterile male and wild fly catches indicated that the released sterile males aggregated in the same sites than wild mosquitoes (Fig. 2), which is a prerequisite for success in an SIT program (25). A maximum ratio of 0.8 sterile to 1 wild male was obtained in the release area (Figure 3). The proportion of unviable eggs collected with 40 ovitraps in the release area was significantly greater as compared with that of a neighbouring control area where no mosquitoes were released ( $p < 10^{-3}$ ). This indicates that the released males were able to compete with wild males, mate with wild females, and transfer their sterile sperm inducing sterility in the native female population. A Fried competitiveness index of 0.26, 95%CI [0.05-0.72] was estimated which compared

favourably with an index of  $< 0.06$  that was observed for the ground-released RIDL *Ae. aegypti* males (14).

**Figure 3:**



Induced sterility and sexual competitiveness of sterile male *Aedes aegypti* released from an UAV-operated release system (b). (A) Temporal dynamics of the sterile to wild male ratio, and rate of viable eggs in the release and non-treated areas. (B) Estimation of the Fried index from 1000 bootstraps in the distributions of sterile to wild male ratios in traps and viable eggs rates in ovitraps in the release and non-treated areas (see SI for details). The Density corresponds to the percentage of the simulations for a given value.

Prior to the release experiments, the Moscamed team was engaged in several public relations activities in the release area which resulted in an overall good acceptance of the drone releases by the general public (see SI for details). The data of this trial indicate that releasing sterile *Aedes* mosquitoes from an UAV platform is feasible with a uniform dispersal of sterile males in the field and a homogeneous sterile to wild male ratio as a result. This is an important outcome especially in view of the low dispersal capacity of *Aedes* mosquitoes. To obtain the same coverage using ground releases would have required a release site every 80 m taking into account the observed median dispersal distance. Releases from the ground in

the required 63 release sites would have necessitated 2 field staff, a vehicle and two hours of work. The UAV release system used in this trial could cover much larger areas by replacing the battery and release cassette more frequently (every 20-25 minutes given the autonomy of the drone at the speed of 10m/sec used in this study), or by using several UAV's that would fly in an echelon formation. The release system might also be mounted on a motorcycle or a bicycle for ground releases in an urban setting. Further improvements to the system are currently under development, i.e. whilst ensuring the same autonomy, the mosquito load may be doubled (100,000), the total weight would remain below 2 kg and a parachute could be added to the system to operate safely in urban areas (26). In addition, improvements will be needed with respect to insulation to ensure a stable temperature below 10°C throughout the flight (Figure S10). The use of an UAV-based system for the aerial release of mosquitoes will significantly reduce the operational release costs. For example, in an IIT-SIT trial against *Ae. albopictus* in China, the cost of releasing from the ground was estimated at 20 USD/ha/week, which could be reduced to an estimated 1 USD/ha/week using a drone (9). Irrespective of the size of the target areas, UAV's might be a good substitute for ground releases to mitigate some of the limitations of ground releases, i.e. no uniform distribution of the sterile males due to the point releases and accessibility of some sites.

In the future, it might even be envisioned that chilled adult mosquitoes are irradiated when already packed into the release cassettes that could then be shipped using courier services from production to release sites within 48 h (27). This would make the technology even more cost effective, as it would abolish the need for costly emergence and release centres in the target areas.

**Table 1.** Main characteristics of the sterile male *Aedes aegypti* released in Carnaíba do Sertão, Brazil. Each row represents a series released separately with a different colour. Year 2018.

Release pattern	Date of Release	Colour	Number released	Recapture rate (%)	Number recaptured	Repeat	Survival rate	Median distance
Ground	March 21	B	9600	1.30	125	1	0.20 (0.96)	97
Ground	March 24	BY	7200	1.90	137	2	0.63 (0.59)	68
Drone_50m_stationary	March 21	O	9600	0.27	26	1	NA	117
Drone_50m_stationary	March 24	OY	7200	0.28	20	2	0.82 (0.48)	148
Drone_100m_stationary	March 21	G	9600	0.05	5	1	NA	158
Drone_100m_stationary	March 24	GY	7200	0.08	6	2	NA	148
Drone_100m_path	March 21	P	50700	0.27	138	1	0.45 (0.80)	NA
Drone_100m_path	March 24	PY	49000	0.42	207	2	0.64 (0.74)	NA
Drone_100m_path	March 27	Y	65700	0.27	175	3	0.70 (0.39)	NA



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## **Supporting Information:**

### **Materials and Methods**

#### Study area and the Moscamed program

Moscamed Brasil is a non-profit organization, based in Juazeiro city (Bahia, Brazil), and operating since 2005. This facility has been working on the implementation of a pilot trial for the control of *Aedes aegypti* since 2011 in different rural and semi-urban areas in Bahia. Carnaíba do Sertão village was selected as a target site to perform a new Sterile Insect Technique (SIT) pilot project. This project started in 2017, and it has support from the National and local authorities, including the local vector control authorities and local community leaders, who participated in previous project activities. Carnaíba is located in Juazeiro (Bahia - 9°35'37.48"S, 40°25'7.17"W) and its population is around 3100 residents in an area of ~51 ha. It is a typical rural area surrounded by native vegetation (Bioma Caatinga) and crops, which provides ecological isolation by reducing migration of *Aedes aegypti*. The mean annual rainfalls are around 400 mm, with a rainy season occurring between November and April. Sanitation and water supply systems are precarious with several open drains, cisterns, tanks, and other types of reservoirs of the community available as mosquito breeding sites. A manageable size, presence of a vector population, adequate topographic surroundings, and consent from the local community and authorities were the essential criteria used for the selection of this area for the present study. The vector surveillance activities using ovitraps are ongoing in this area since 2017 which facilitated the interactions with the local community.

#### Community engagement

Before the trial, Moscamed team contacted the Bahia Municipality Health Public Secretary to inform about the objective of these aerial releases, its support, and achievements. Two meetings were carried out with the Health Surveillance Superintendence to share the goals of the trial with supervisors and discuss entail points to access people's knowledge about the use of SIT for mosquito control. They contributed with crucial recommendations for the best approach to obtain local community agreement to perform the study. Besides the authorities, the vector control agents and local community leaders were trained in communication and stakeholder engagement, so that they would be able to support and disseminate the trial objectives among the local community. Their role was critical to set-up appropriate locations for monitoring traps used in this study. Most of the community engagement activities took place locally to clarify as much as possible the trial steps, such as visiting households for monitoring, and included the distribution of leaflets. Also, a TV interview by the local press took place, with Moscamed representative and researchers, to provide information about the study. All these activities allowed a high acceptance of the use of drone releases by the community.

#### Mosquito colony rearing:

##### Laboratory strain: Insect Pest Control Laboratory, Seibersdorf

The strain of *Aedes. aegypti* used in all experiments described here in was sourced from Juazeiro, Brazil and transferred to the Food and Agricultural Organisation/ International Atomic Energy Agency (FAO/IAEA) Insect Pest Control Laboratory (IPCL) in Seibersdorf, Austria by Biofabrica Moscamed, Brazil. Adults since 2010 without further colony regeneration. Adults are maintained in a climate controlled insectary (temperature (T)  $27 \pm 1^{\circ}\text{C}$ , relative humidity (RH)  $70 \pm 10\%$ , photoperiod (L:D) 12:12,

with two one-hour twilight periods simulating dawn and dusk) as described in (1). Eggs were generated for all experiments and hatched based upon standardised guidelines developed at the IPCL (2). Larvae were reared in plastic trays (40 x 29 x 8 cm) containing 1 – 1.5 litres of deionized water at a density of roughly 1500 - 2000 first instar (L1 ) per tray and were fed daily with IAEA diet developed and described in (1, 3, 4). Pupae were sexed mechanically using a Fay-Morlan (5) glass plate separator as redesigned by Focks (John W. Hock Co., Gainesville , FL, USA (6)), prior to further examination under a stereomicroscope for increased accuracy. Adults were maintained in plastic Bugdorm cages (30 x 30 x 30 cm, Taiwan) unless otherwise stated with continuous access to a 10% sucrose solution. All experiments were carried out on 3 – 4 day old adults to reflect the likely age of release, unless otherwise stated.

Field strain: Moscamed, Brazil

The strain of *Ae. aegypti* (MBR-001) used in the present study was obtained from field material (eggs) collected in the Carnaíba neighborhood (09°35'40"S, 40°24'58"W), Juazeiro city, Bahia State, northeast Brazil. Sterile males were reared in a climate-controlled insectary at the mass-rearing Unit of Moscamed Brasil (T 28 ± 1°C, RH 80 ± 10% and a photoperiod of L10:D14h). Larvae were reared in plastic trays (51 x 30.3 x 9.7 cm), at a density of 1 larvae/ ml in 3 liters (L) of mineral water. Larvae were fed daily with a solution of the IAEA 2 liquid diet (4% w/v) until pupation (3). Pupal separation was carried out by size (female pupae > male pupae > larvae) using a glass plate separator (Moscamed Brasil model) (4-6) as described by (7). Pupae were kept in trays containing mineral water in a climate-controlled insectary until irradiation (T 27 ± 1°C, RH 70 ± 10%, photoperiod of L10:D14h).

### Irradiation protocols

#### Seibersdorf

*Ae. aegypti* pupae were irradiated at  $90 \pm 5$  Gy between 36 and 48 hours old, with all water removed, inside a self-contained  $^{60}\text{Co}$  Gamma Cell 220. The actual dose received was measured with a dosimetry system using Gafchromic MD film (International Specialty Products, NJ, USA) (7).

#### Juazeiro

Male pupae sterilization was carried out at the Moscamed Brasil using RS 2400 X-ray machine (RadSource, Suwanee, GA, USA) with a 125kV voltage, an 18mA current, and with a dose-energy ratio of  $0.0207 \text{ Gy kW}^{-1} \text{ s}^{-1}$ . Male pupae (30-36 hours-old) were irradiated with a dose of 35 Gy leading to a sterility rate  $> 99\%$  in these conditions. The pupae were placed in 12 well cell culture plates (diameter 2.14 cm/well, area  $3.66 \text{ cm}^2/\text{well}$ ) containing a small amount of water (1.5 ml). The plates were placed in a horizontal position inside of a polyfoam prototype (diameter 16.7 cm, length 11.7 cm) developed in the workshop of Moscamed Brasil to position pupae in the most central part of the irradiation cylinder (diameter 17.5 cm, length 14 cm) to minimize dose variation. After irradiation, the pupae were transferred to laboratory cages (30 x 30 x 30 cm) and kept in a climate-controlled insectary until adult emergence ( $T 27 \pm 1^\circ\text{C}$ ,  $\text{RH } 70 \pm 10\%$ , photoperiod of L10:D14h). Sterile males were provided with 10 % sucrose solution *ad libitum*.

### Quality control – flight test device

A flight test device (FTD), which aims to evaluate the flight ability of an adult mosquito, was created during the project (8). The FTD consists of a series of 40

transparent acrylic plastic (Polymethyl methacrylate - PMAA) flight tubes, surrounded by a larger PMAA tube. The first two series of tubes are housed within a third PMAA tube of greater size which serves as a containment box after mosquitoes escape the flight tubes. Mosquitoes are blown into the FTD via a mouth aspirator and are given a period of 2 hours to escape. Afterwards, the FTD is chilled at 4 °C and the number of adults that remain at the base of the flight tubes and those that have escaped are counted. Flight ability is calculated by dividing the number of adults which escaped by the total number put into the flight tube. An average is then calculated across 2 repetitions. A series of verification experiments confirmed that flight ability was an accurate predictor of male mosquito quality. Thus, we used flight ability as an indicator of quality when conducting our laboratory tests related to the design and development of our prototype release mechanism. Further information regarding the FTD including schematic drawings, full dimensions and verification experiments results can be found in(8).

## Compaction

### *Defining the maximum tolerable height of compaction*

The aim of this experiment was to measure the damage caused by the compaction of the mosquitoes and to ascertain the maximum pressure tolerable. This parameter was critical when designing the storage container for the release mechanism. We used a cylindrical tube (height 10 cm, diameter 4 cm) with holes (1 cm diameter) cut vertically at 2 cm intervals. 15 Bugdorm cages of male *Ae. aegypti* (approximately 50 000) were transferred to the cold room (4°C) and left for 10 minutes until immobile. The cylinder was placed on a scale and tared. All cages (except one which served as a control cage) were emptied into a larval rearing tray and mosquitoes transferred to the cylinder via a plastic funnel and a timer set for 2 hours. Following this 2 hour period of immobilization

and compaction, a mouth aspirator was used to remove small samples (approximately 200) of mosquitoes via the pre-cut holes at different heights of 3, 5, 7 and 9 cm (equivalent to 0.76, 1.27, 1.78 and 2.29 g/cm<sup>2</sup> respectively). All samples, in addition to a sample from the control cage, were taken to the laboratory and split into 2, thus generating 10 batches of adults and flight ability tests were conducted in the flight test device (FTD).

#### Active compaction

In the event that the phase change material (PCM) was to fail during transportation to the field site and or during the actual release and the temperature were to rise above that necessary to maintain immobilisation, we were keen to know the impact this would have upon the compacted sterile males whilst held in the storage container. A Bugdorm containing approximately 3000 *Ae. aegypti* males were immobilized for 10 mins inside a cold room (4°C). A cylindrical tube of 8 ml (height = 5.5 cm, diameter = 1 cm) was filled with immobile males, closed with a small square of mesh and secured with an elastic band. The volume of the tube allows approximately 1000 adult males to be contained. This was repeated with the remaining males, generating 3 tubes of immobilized males. The tubes were transferred to ambient laboratory conditions (described above) and a timer begun. After 10 minutes, one tube was emptied into a standard Bugdorm cage and again after 20 and 30 minutes.

#### Chilling

#### Wake up times

In order to determine the altitude at which the drone will conduct an aerial release, it was important to know how long the sterile mosquitoes would need to “wake up”



following immobilisation for  $x$  amount of time at  $y$  temperature (in addition to the speed at which they fall when immobile, to be detailed later). Thus, we conducted an experiment at 3 temperatures across the range that we anticipated to store the mosquitoes during transport and release (6, 8 and 10°C) and for different lengths of time (1 to 4 hours) in order to ascertain how long it takes them to recover and regain the muscle activity necessary to fly. Batches of 1000 male *Ae. aegypti* were immobilised and transferred to a small plastic tube (height = 5.5 cm, diameter = 1 cm) and transferred to a climate chamber pre-set to each of the temperatures to be tested. After each time interval, 2 tubes of immobile males were removed and emptied into separate Bugdorm cages. A timer was begun and when approximately 75% of the males had regained flight the timer was stopped. An average was calculated across the 2 repetitions for each temperature and duration.

#### Release mechanism speed

In order to further fine-tune the homogeneity of release, according to roughly how many mosquitoes we intended to release per hectare (ha), we investigated 2 different rotation speeds of the cylinder of 1 and 3 repetitions per minute (rpm). Cages totalling approximately 50 000 male *Ae. aegypti* were immobilized in the cold room (4°C) with half loaded into the storage container above the cylinder. We tested both 1 rpm and 3 rpm (half of the batch of 50 000 mosquitoes in each test) and video recorded each test to assess the homogeneity of each release speed. One cage of mosquitoes was set aside to serve as control. Samples were taken from each speed for flight ability tests, from the top, middle and bottom layers of mosquitoes within the storage container, in addition to a sample from the control cage.

### Drop speed of mosquitoes

Another factor in determining the altitude at which mosquitoes are to be released was the speed at which they typically fall. Thus, we implemented a video capture set, which comprised a high performance industrial video camera (IDS camera), a metric ribbon resting on the wall (2 meters in length), and a white led light high power focus (40W). The optics used for this test had a maximum focal length of approximately 55cm and a 35° field of view and thus only allowed for focusing on a 35cm section of the metric ribbon. Therefore, it was decided to only focus on the last 35cm of the metric ribbon (closest to the floor). For the analysis, we used different male mosquito samples (*Ae. aegypti*) which had previously been frozen to kill them. We selected three different individuals that showed small differences in size. All samples were dropped repeated from four different heights: 50, 80, 110 and 140 cm.

### Wind resistance test

The aim of this experiment was to investigate the effect of exposure to various wind speeds on the flight ability of sterile male mosquitoes. Wind is a natural phenomenon and when releasing sterile male mosquitoes by air, it may be amplified by the movement of the drone in flight. Thus, it was crucial to ascertain if there was a wind speed above which significant damage occurred to the sterile males. It also allowed us to determine what speed we should conduct the aerial releases at. The wind tunnel was composed of a Plexiglas tube (diameter 150mm) with a powerful fan at one end, adapted from a basic garden leaf blower. The dropping tube, the point at which sterile males are introduced into the wind tunnel, was placed vertically at a distance of 10 cm on the laminar setup with the end of the tube being at the centre of the wind tunnel. At the opposite end, a Bugdorm cage (30 x 30 x 30 cm) was used to catch the mosquitoes

after they are blown through the wind tunnel. An anemometer was inserted through a small slot on the top of the Plexiglas tube, close to the dropping tube to enable the speed of the leaf blower to be adjusted until the correct speed was reached. Four speeds were tested in total, 7, 11, 15 and 19 meters/ second (m/s) with control samples simply dropped into the wind tunnel when the leaf blower was switched off (0 m/s). Several Bugdorms of three day old sterile male *Aedes aegypti* were taken to a cold room (4°C) and left for a period of 10 minutes until immobilization had occurred. Adults were gently tipped out of cages into a plastic tray and then further transferred to 10 falcon tubes (15 ml) via a funnel, until a volume of 7.5 ml was reached in each tube. This volume equates to approximately 1000 adult male *Ae. aegypti*, when no compaction is used. One falcon tube of adults was introduced into the wind tunnel for each of the 4 tested wind speeds with 2 repetitions allocated to each speed and 2 control samples. A subsample of approximately 100 adults were taken from the Bugdorm following each wind speed test and quickly transferred to the flight test device (FTD) to calculate flight ability (as described previously).

#### Competitiveness analysis in large cage

It was critical that we investigated the impact our release system had on the competitiveness of sterile male *Ae. aegypti*. Thus, we simulated a full run through, mimicking an actual aerial field release and in parallel, simulated a ground release, prior to calculating the competitiveness index (CI). *Ae. aegypti* were reared as previously described above, separated into batches of males and females and further screened under a stereomicroscope to ensure the accurate sex separation of 30 000 males and 1200 females. 29 000 male pupae were irradiated as previously described, at  $95 \pm 5$  Gy aged  $40 \pm 4$  hours old. The remaining 1000 male pupae were not subject to

irradiation to serve as fertile males and caged in batches of 100. Female pupae were also caged in groups of 100. For both females and fertile males, 2 batches of 100 served as back up adults. Sterile male pupae were caged in batches of approximately 3000 (volumetric estimation). On day 3 post-emergence, cages of females and fertile males were adjusted back to batches of 100 to compensate for failed emergence and mortality from the back up cages. All cages of sterile males, in addition to the 3 cages of 300 sterile males (ground release), were transferred to a cold room ( $4 \pm 1$  °C) for a period of 10 minutes until immobilization occurred. All cages were emptied into a plastic larval rearing tray ( $30 \times 40 \times 7$  cm) and carefully transferred to the storage unit of the aerial release system. The storage unit is designed to hold 50 000 mosquitoes and thus was only at half capacity, as in total only 900 sterile males were required for this experiment. The rearing tray was placed underneath the ejection mechanism to recollect the sterile males after they passed through the aerial release system, which was set to operate at the speed chosen for the actual aerial releases in Brazil (3 repetitions per minute). Once all sterile males had passed through the release system, 3 batches of 300 were counted out and transferred to a small plastic container (100 ml) and closed. The 3 cages of ground released sterile males were also transferred to such containers. All containers were returned to the laboratory to be transferred to allocated large Bugdorms ( $60 \times 60 \times 60$  cm) as follows: 2 sterile controls (100 sterile males), 2 fertile controls (100 fertile males), 3 ground release cages with sterile males which did not pass via the release mechanism (100 fertile males and 300 sterile males) and 3 aerial release cages, with males which did pass via the release mechanism (100 fertile males and 300 sterile males). Once all males had been assigned to their cages, 100 females were added to each of the 10 cages. A period of 72 hours was given for mating to occur after which females were recollected from each of the 10 cages and transferred to 10 new

Bugdorms (30 x 30 x 30 cm). Bloodmeals were offered daily for the next 2 days and an egg cup placed in each Bugdorm of females. After 72 hours, the egg papers were collected and dried for a period of 2 weeks to allow the eggs to mature. Each paper was then hatched and after 24 – 48 hours, the number of larvae in each tray was calculated. Additionally, the egg paper was viewed under a stereomicroscope and the number of hatched and unhatched eggs counted to calculate the hatch rate.

The SIT relies on the release of mass-produced male flies that are sterilized by ionizing irradiation. Consecutively, wild female flies are in turn sterile after mating with sterile males. A good competitiveness of the released sterile males is crucial to warrant the success of this technique (10, 11). The evaluation of this competitiveness was based on the assessment of the impact of sterile males on female fertility (12). Fried (1971) defined a competitiveness index, called Fried's index that can be calculated with the following formulae:

$$F = \frac{\frac{Ha - Ee}{Ee}}{R}$$

where *Ha* is the natural fertility of wild females and *Ee* the observed fertility rate under a given ratio of sterile over wild males, *R*. This formula can be applied when the residual fertility of males can be neglected, which was the case for a 90 Gy dose.

#### Simulated release within laboratory conditions

Prior to conducting the mark-release-recapture (MRR) study in Brazil, we carried out one last laboratory test with the aim to execute a simulated release with all predefined parameters. Cages totalling approximately 25 000 sterile male *Ae. aegypti* were

immobilized at 4°C in a cold room for 10 minutes prior to being transferred to the storage container, with one cage left aside to serve as controls. The container was fully surrounded by phase change material (PCM) during this experiment. The storage container was connected to the release mechanism and placed inside a Bugdorm. A climate chamber was pre-programmed to reflect likely environmental conditions within the field (35°C and 80% relative humidity). The release mechanism was connected to the software which controls the release when connected to the drone during an actual aerial release. The Bugdorm was placed inside the climate chamber and the door to the release mechanism removed. The Bugdorm was gently shaken to simulate the drone commencing flight. As the storage container was only at half capacity, we chose to set the speed of release at 1 rpm thus the time taken until all mosquitoes were ejected from the storage container was approximately 15 mins (similar to the time it will take at 2 rpm to release a full container of sterile males). 2 samples of approximately 100 mosquitoes were removed from the Bugdorm, in addition to 2 control samples and flight ability tests were performed.

#### Marking protocol

Sterile male mosquitoes were dusted with the equivalent of 0.001 g or 1 mg/ 100 adult males in a 100 ml cylindrical container with pigments from the Dag Glo series. For the MRR, we marked in batches of 2400, thus size of the container and the quantity of dust was also increased (1 L container and 24 mg) of dust. To ensure dust adhered to the walls of the dusting container, the inside surfaces were rubbed with sandpaper to create a rough as opposed to smooth surface. The dust for each container was weighed on an analytical balance and then transferred to the container and closed (dust colours and combinations can be found in Table S2.). The container was shaken vigorously to coat

the inner surfaces evenly. All containers were taken to a cold room (4°C) and left to acclimatize. Cages of 2400 adult *Ae. aegypti* were then transferred to the cold room for immobilization for 20 minutes. Each cage was then emptied into a pre-dusted container and the lid closed. The container was then rotated for 30 seconds (equating to approximately 25 full rotations) to coat the sterile males uniformly. Dusting took place late in the evening (no earlier than 6pm) which equated to around 12 hours before each release occurred the following day. Sterile males were left immobilized in the dust containers overnight with the cold room temperature raised to 8°C. The following morning, dusted mosquitoes were transferred to storage containers according to their dust colour and packed into a cool box for transportation to the field site.

#### Mosquito Release Mechanism design

We designed a release mechanism including mechanics, electronics and software. The mechanism mounts on a drone and enables aerial release of mosquitoes. The main parts of the release mechanism are: (1) storage unit consisting of a canister that keeps mosquitoes at cold temperatures surrounded by insulation, (2) an ejection mechanism featuring a rotating cylinder that brings mosquitoes from the storing canister to the outside, (3) a release area where mosquitoes fall onto and then slowly enter the wide open, (4) onboard electronics featuring sensors and cameras to control and monitor the state of the mechanism and mosquitoes.

The storage unit or holding canister was designed to contain 50,000 mosquitoes. In order to keep the insects at the target temperatures, we put phase change materials (PCM) with a target temperature of 4 °C in the canister walls. The canister was placed in an insulation box made of Styrofoam to minimize heat exchange. The whole storage

unit featuring the canister and insulation box could then be loaded into the ejection unit. This enabled us to load the release mechanism multiple times while in the field without the need to remove any parts from the drone.

The ejection mechanism consists of a rotating cylinder connected to a stepper motor embedded in a structure. This mechanism was developed for other fragile insects within the ERC REVOLINC project (PCT/EP2017/059832). The cylinder has 6 discrete holes that each can take up around 800 mosquitoes. Hence a full cylinder turn should release around 5,000 mosquitoes. The stepper motor controls the rotation of the cylinder with high accuracy and high torque. The motor can be set to various speeds. We found that values between 1-3 RPM are optimal, leading to release rates of 5,000-15,000 mosquitoes per minute. The structure around the cylinder is built to minimize airflow from the outside to the inside of the canister. In addition, the connection between cylinder and structure is designed in such a way that it is easy to remove the cylinder for cleaning.

The release area is simply an inclined surface where mosquitoes fall onto after transportation through the cylinder. While the cylinder ejects discrete amounts of mosquitoes, the airflow through the release area moves the mosquitoes more gently into the surrounding air, making the release more continuous. Also, the white background on the inclined surface (and a camera pointing at it) allows the user to see and monitor the release using a real-time video stream.

The onboard electronics control is running on a Raspberry Pi 3 (RPi, low-cost mini computer), interfacing the drone (and ground station) with the release mechanism. A



LCD screen is mounted on the drone and gives visual feedback of the onboard control when in the field. The stepper motor is controlled using an STM32 microcontroller and a motor controller shield that receives motor commands from the RPi. In order to monitor the mechanism during flight, we embedded several sensors into the mechanism. Four temperature sensors are mounted at locations outside the mechanism, at the cylinder, at the canister wall and inside the canister. Two humidity sensors measure outside humidity and humidity in the canister. Further, we mounted two cameras to monitor and live-stream the release area as well as the canister load and drone flight. The cameras give direct visual feedback to the user about the release of the mosquitoes.

#### Drone integration

The whole mechanism is embedded on a DJI M600 Pro hexacopter drone using a custom-made holding structure that allows for simple mounting and unmounting. The M600 Pro is a professional six-rotor drone made for industrial applications that comes with a range of DJI technologies, including a robust flight controller and a strong transmission system (up to 5km long range transmission). It enables a flight time of 30-35min when equipped with a payload of 1-2 kg. Also, it features a dust-proof propulsion system with actively cooled motors making it reliable and robust during extended missions. The M600 Pro can be extended with third-party hardware components and is fully compatible with the DJI Onboard SDK and Mobile SDK to build software adapted for our own purpose.

#### Ground station Software

In order to run mosquito release missions autonomously, we developed a custom Android- based app that allowed for efficient planning and running of such missions. The main features of this ground station app were planning of flight route, speed and altitude, setting release points and rates, uploading a mission to drone, running a mission autonomously, monitoring drone state, mechanism state, sensor values and camera live- stream. Missions could be saved and loaded for repeating the exact same missions. In addition, KML files featuring GPS positions could be imported, allowing to plan the flight route using standard GIS tools.

### System Calibration

In order to calibrate our system for a target mission, we mainly needed to set a flight route, the release rate of the mechanism as well as the flight altitude. The flight route is best set as a regular polygon pattern above the target area. The sidelap between each leg of the flight (distance between release lines or swap) is mainly related to the dispersal of the mosquitoes. Assuming a dispersion of around 50 m a priori, we chose the side lap between the flight legs to be 80 m. The release rate per area depends on the turning speed of the cylinder and the flight speed. Using the formula below we could derive a cylinder speed and flight speed for a target release rate for a given flight route/leg:

$$\text{Release rate per flight line (Mosquito/m)} = 5,000 * \frac{\text{Cylinder speed (RPM)}}{\text{Flight speed (m/s)}}$$

### Mark-release-recapture protocol

Our final study aimed to estimate the dispersal, mortality and mating capacity of sterile male *Ae. aegypti* mosquitoes through mark – release – recapture (MRR) experiments after being released from either the ground or by air in a pilot site in Brazil. The MRR

experiments were conducted within a pilot site situated in Carnaíba do Sertão, Juazeiro, Brazil. A pilot site of 20 hectares (ha) was mapped, with 35 trap locations (Figure 2). The average daytime temperature in this area was 32 °C with a monthly precipitation of 101 mm (based on March averages). The MRR study involved releasing sterile males irradiated at 35 Gy by X-ray (see irradiation section above for detailed protocol), in an open field setting. 3 releases were conducted within a 7 day period (Table 1). Aerial releases involved our prototype release mechanism attached to a DJi Matrice pro 600 drone (Figure 1). Aerial releases occurred in 2 ways. Firstly, sterile males were released in the centre of the pilot site at altitudes of 50 and 100 m with the drone hovering in a stationary position (Figure 2). Ground releases entailed adults being released from a container in the same release site were conducted as controls. Secondly, sterile males were released along selected paths at an altitude of 100 m with release lines spaced 80 m apart over all the area. Sterile males were marked according to their release type and release day (for detailed marking protocol see marking method above).

Prior to the day of the first release (20 March), 35 baited BG-sentinel traps were positioned in the MRR pilot site, referring to a rectangular area of 20 ha (Figure 2), with a density of 1.75 traps per ha. In each of the trapping stations, one ovitrap was set in the vicinity of the BG trap (<50m). 5 ovitraps were also set in a neighbouring control area (at 0.9km from the release area) to measure the natural fertility of *Aedes aegypti* during the same period. In the early morning of March 21<sup>st</sup> 2018, sterile males were released either by air or by ground as described above. The following day (March 22<sup>nd</sup>) beginning early afternoon (12:00 pm to 14:00 pm), traps were inspected and the samples collected withdrawn and brought to the laboratory. All mosquitoes caught were

give n an identification code referring to the relevant station in order to calculate dispersal capacity. Collected adults were immediately placed in an insulated storage container. Upon returning to the laboratory, all samples were transferred to a freezer (-20°). The following day (March 23rd) and after each collection day thereafter, field collected samples were analysed, classified and data stored. Samples were screened for colour under a UV-light stereomicroscope. Collections were made by 2 teams of 4 people, with each team responsible for monitoring 17 or 18 traps. Traps were monitored daily for a period of 14 days after each release (thus until April 10th for the third and final release). Eggs collected were dried for 7 days and then hatched. Non-hatched eggs were bleached to check for the presence of an embryo. Release and recapture data were geo-referenced using a Global Positioning System device. All coordinates were entered into a Geographical Information System to calculate the distances between release and each recapture site.

### Data analysis

Re-capture rates were compared using proportion comparison z-test and difference between release mechanism were tested using pairwise proportion test between each mechanism. We used a Kruskal Wallis rank test to compare the overall and we then used some pairwise tests to compare each release mechanism correcting the p-value to account for multiple comparison. Binomial linear mixed effect models were used to analyse the impact of the various treatments on escape rates from the flight test device (response variables). The treatment regimens were then used as fixed effects and the repetitions as random effects. The significance of fixed effects was tested using the likelihood ratio test (13, 14). In order to obtain a confidence interval for the estimate of the Fried Index, we used a non-parametric

bootstrap approach (15). The data on fertility and ratio of wild male over wild one were resampled without replacement and for each set of resampled data we computed the Fried index (1,000 simulations). Assuming a symmetric distribution, we used the basic percentile method to get 95% confidence interval.

## **Results**

### Defining the maximum tolerable height of compaction

Compaction significantly reduced the flight ability of male *Ae. aegypti* from a height of 7 cm ( $0.178 \text{ g/c m}^2$ ) onwards, with no significant difference in flight ability observed between the control samples and those exposed to 3 or 5 cm or compaction ( $0.76$  or  $1.27 \text{ g/cm}^2$ ) (Figure S1). This finding confirmed an earlier observation using *Ae. aegypti* that 5cm of compaction is the maximum tolerable level that can be imposed upon immobile *Ae. aegypti* (8).

### Active compaction

We observed a 100% recovery of all mosquitoes after 1, 2 and 3 hours of active compaction and thus no further experiments were carried out on active compaction.

### Immobilisation and storage temperatures

The suitable temperature range to conduct immobilization and store sterile male *Ae. aegypti* during transportation to the field and prior to release was identified previously as the range 8- 10°C after testing temperatures between 0 and 10 °C for 2 hours (8).

### Wake up times

The chilling temperature and duration, as well as their first order interaction, significantly impacted the wake up time (Table S1 & Figure S2). The longer and lower the chilling temperature, the higher the wake up time. At 10°C for one hour, the wake-up time of 75% of chilled adult males was around 40 seconds.

### Release mechanism

The flight ability was higher for the cylinder than the conveyor belt ( $p = 0.03$ , Figure S3). Homogeneity of the mosquitoes passing via each mechanism (based upon video footage) was also better for the cylindrical release system which was selected as the final design. After testing four different variations of the cylinder shape and reviewing the video footage, we selected the one which gave the most homogenous release of male *Ae. aegypti* (Figure 1).

Flight ability did not differ significantly between controls and males which passed via the release mechanism at either 1 or 3 RPM ( $p > 0.714$ , Figure S4). Furthermore, the position of the males within the storage container prior to passing via the release mechanism did not significantly decrease their flight ability in comparison to control males ( $p > 0.488$ , Figure S5). For the actual field releases in Brazil, we adjusted the flight speed of the drone to 10 m/s and the cylinder speed to 2 RPM to release approximately 5000 sterile males per ha.

### Drop speed of mosquitoes

Based on this experiment, we found that the maximum free fall speed of *Ae. aegypti*, in a closed space, would have a near upper limit of about 2.5m/s, which ensures a

minimum falling time of 40s at a 100m dropping height. Due to the speed of the drone and climatic conditions, this falling time should be higher in a real scenario. Assuming a wake-up time of the mosquitoes around 40 seconds, flight altitudes of 50 and 100m were thus selected for testing in the field.

#### Wind resistance test

We did not observe any significant impact of wind speed on the quality of sterile males as measured with their flight ability ( $p > 0.09$ , Table S2), as measured in our wind speed test chamber (Figure S6).

#### Competitiveness analysis in large cage

Results of a Welch Two Sample t-test ( $t = -0.036$ ,  $df = 3.998$ ,  $p\text{-value} = 0.973$ ) indicated that males which had passed via our prototype release mechanism in a simulated aerial release were of equal competitiveness as males exposed to a simulated ground release (Figure S7). The competitiveness index of males which underwent a simulated aerial release was 0.66 (SD 0.07) in comparison to those exposed to a simulated ground release (0.67, SD 0.07). Hatch rates from cages with only sterile males from simulated ground or aerial release were on average 1.12 and 1.10% respectively, corresponding to their residual fertility.

#### Simulated release within laboratory conditions

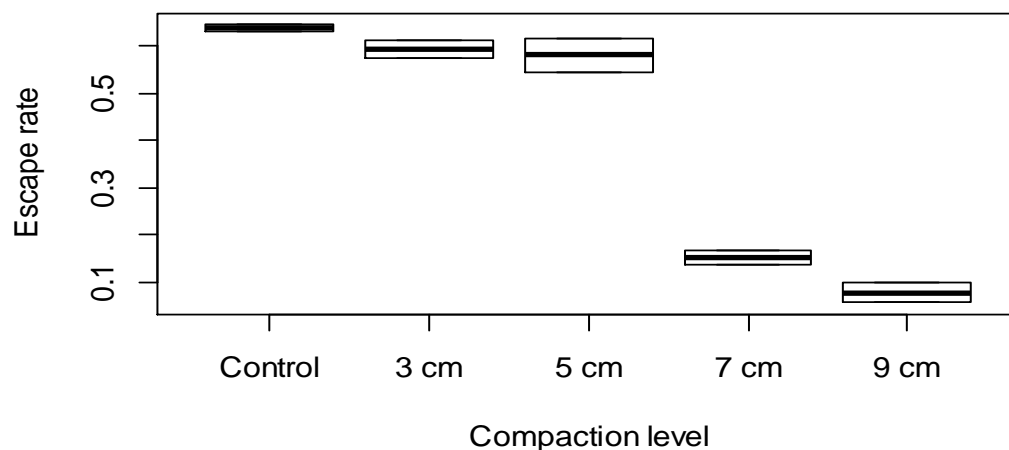
In laboratory conditions, sterile males that passed via the release mechanism in a simulated aerial release did not differ significantly in flight ability ( $76.01 \pm 1.01\%$ ) in comparison to controls males that did not, thus simulating a ground release ( $78.04 \pm 0.54\%$ ,  $p = 0.837$ ).

### Mark-Release-Recapture

Although not easy, we successfully discriminated the various series of released males using high magnification in the visible spectrum (Figure S8). The mortality rates of the different series released in the field were similar between treatments ( $p>0.46$ , Table S3), i.e. we did not find any differences between point and path releases or between release altitudes. The temperature was not maintained below 10°C throughout the flight but it remained below 18°C which did not cause a strong reduction of the quality given that the flight duration was below 13 minutes (Figure S9). All other results are presented in the main text.

## Figures

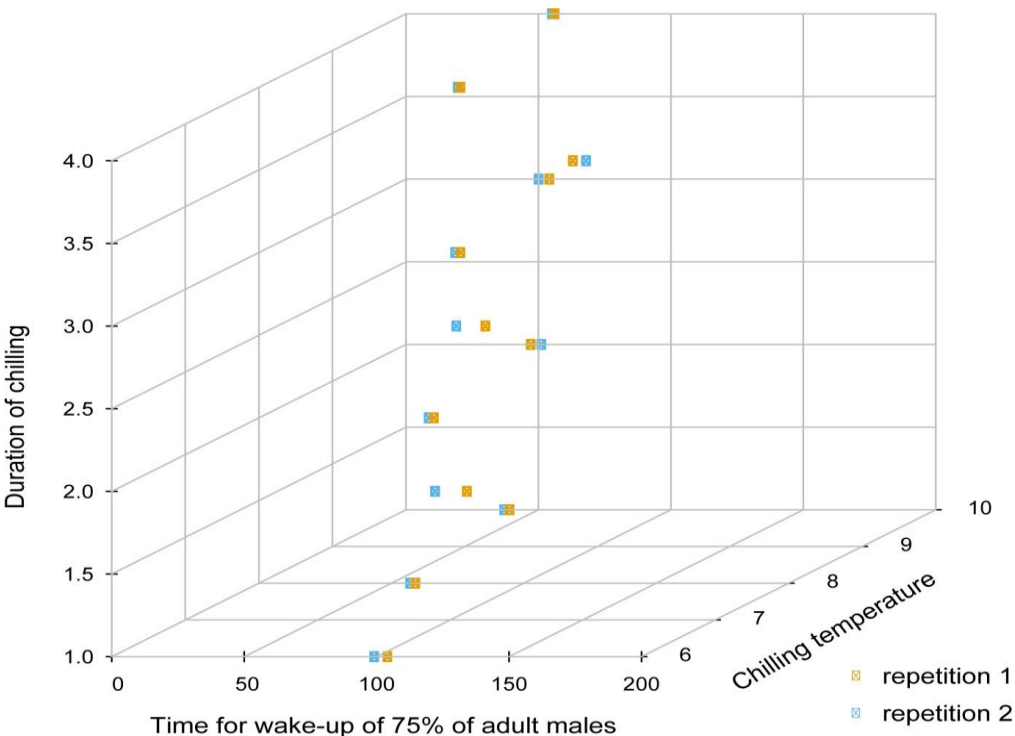
**Figure S1.**



Flight ability results of male *Aedes aegypti* following two hours of immobilization at 4°C under various levels of compaction

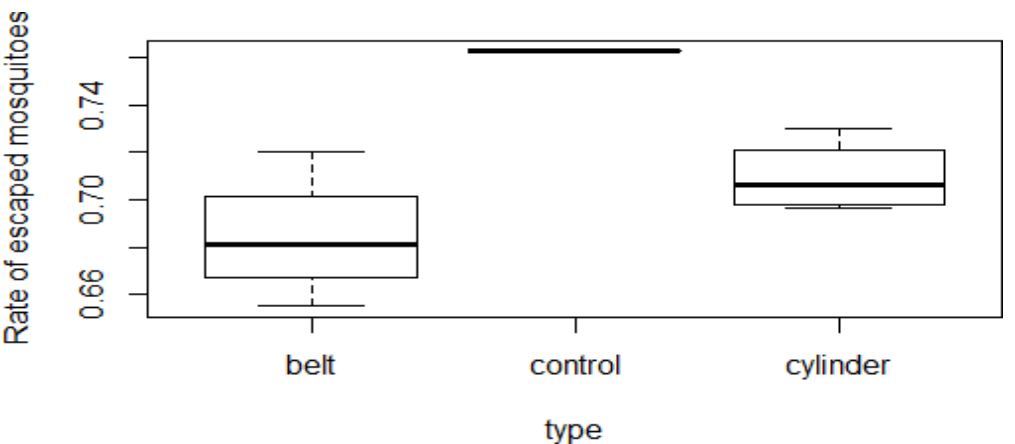


**Figure S2.**



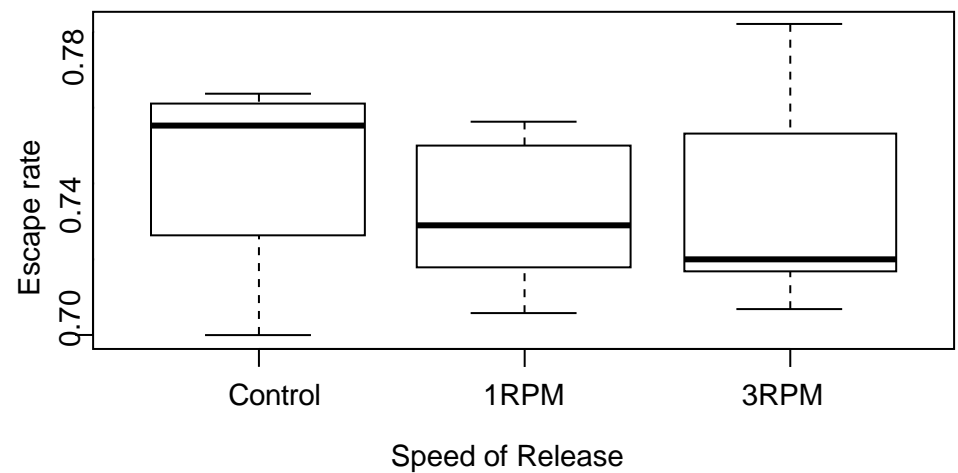
The average time taken (secs) for 75% of adult male *Ae. aegypti* to regain flight ability following immobilization at 6, 8 and 10°C for 1 – 4 hours.

**Figure S3.**



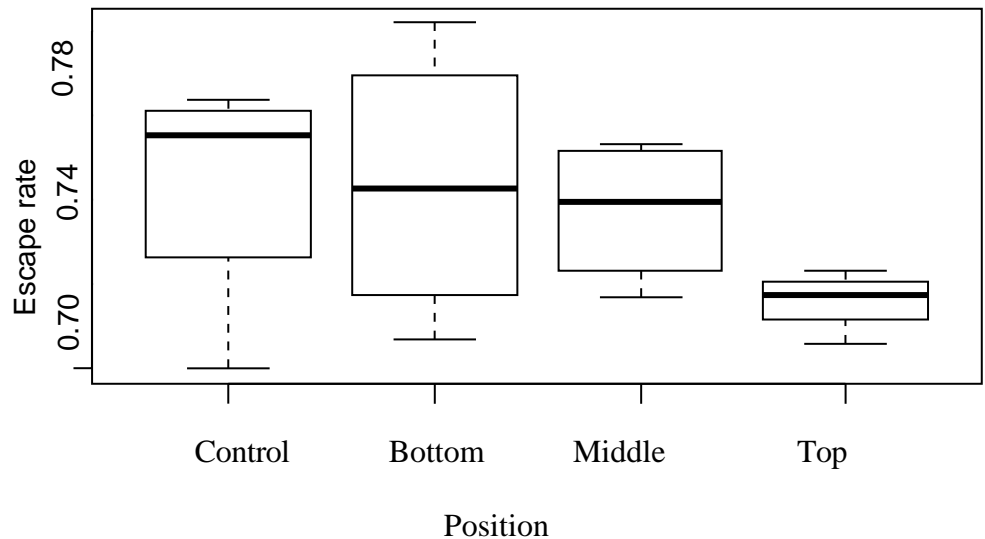
Flight ability results of male *Aedes aegypti* after passing through two prototype release mechanisms versus a control sample.

**Figure S4.**



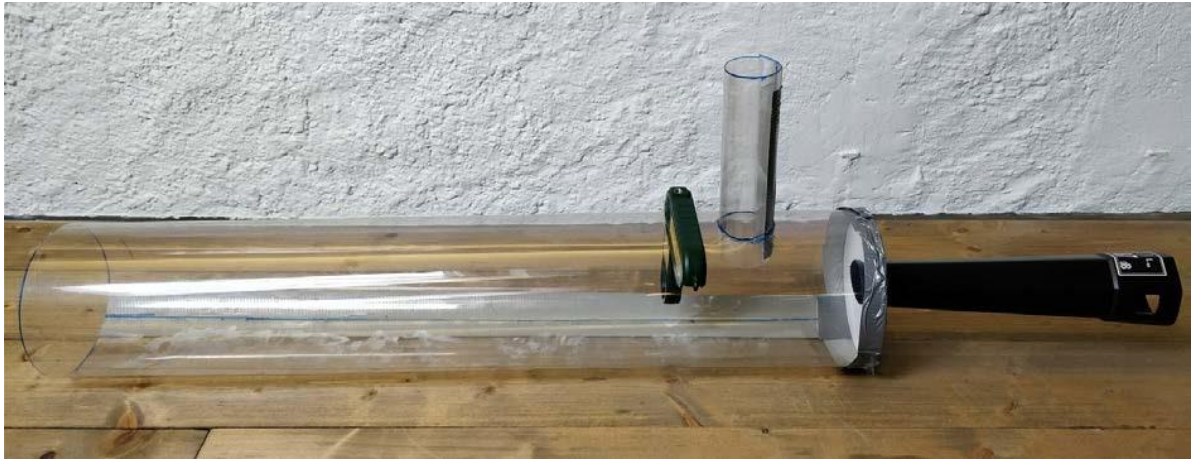
Flight ability of male *Aedes aegypti* after passing through the cylinder release mechanism at different speeds (1 or 3 revolutions per minute (RPM)).

**Figure S5.**



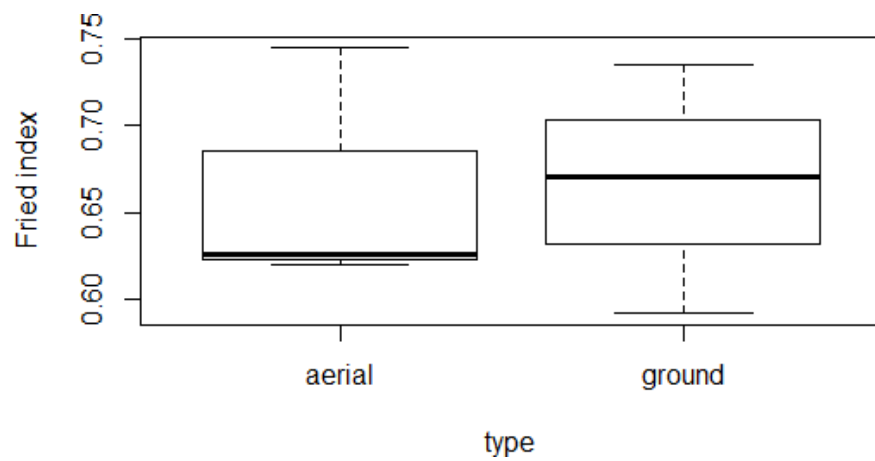
Flight ability of male *Aedes aegypti* after passing through the cylinder release mechanism depending on their position in the canister.

**Figure S6.**



Wind speed test chamber.

**Figure S7.**



Fried competitiveness index of male *Aedes aegypti* released using our prototype aerial release system or by ground in large cages at the laboratory.

**Figure S8.**

A



B



C



D



E



F



G



H

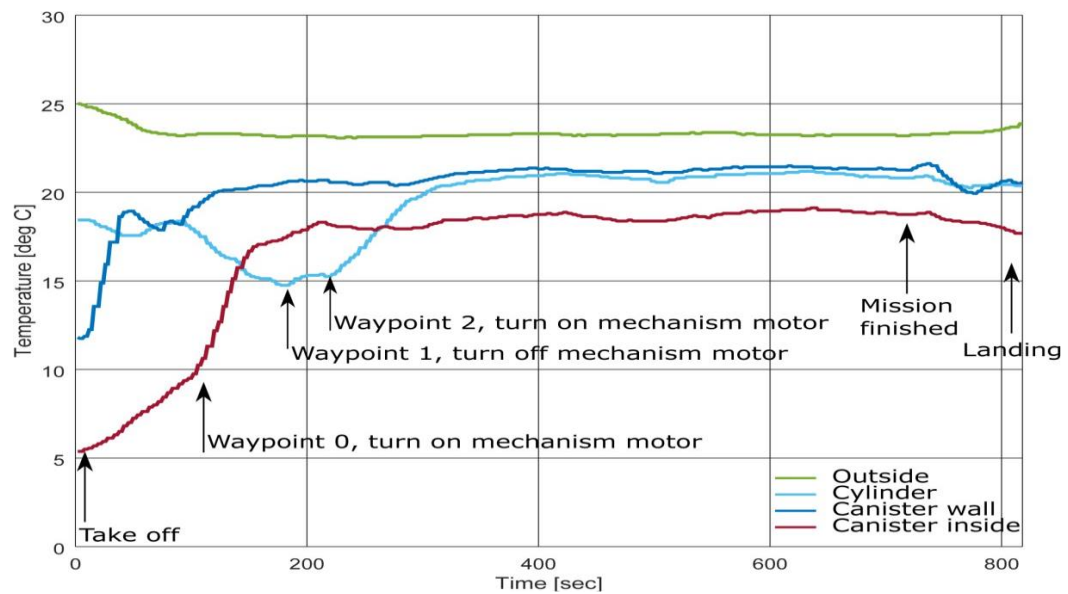


I



Differentiation of sterile males from wild flies using fluorescent dust. A (blue) first ground release. B (green) first stationary drone release (100m). C (orange) first stationary drone release (50m). D (pink) first drone release (100m flight path). E (blue-yellow) second ground release. F (green-yellow) second stationary drone release (100m). G (orange-yellow) second stationary drone release (50m). H (pink-yellow) second drone release (100m flight path). I (yellow) third drone release (100m flight path).

**Figure S9.**



Dynamics of the temperature inside the release system during a flight. The flight altitude was 100m and correspond to the path release of March 21 2018 described in Table 1 in the main text.

## Tables

**Tables S1.**

Fixed effects	Value	Std. Error	DF	t-value	p-value
Intercept	35.75	5.1452	17	6.9482	0
Temp 8 °C	18.00	7.2764	17	2.4738	0.0242
Temp 6 °C	42.50	7.2764	17	5.8408	0
Chilling duration	5.55	1.8788	17	2.9541	0.0089
Temp 8°C : Chil. dur.	0.70	2.6570	17	0.2635	0.7954
Temp 6°C : Chil. dur.	17.70	2.6570	17	6.6617	0

Fixed-effects coefficients of a Gaussian model of the impact of temperature and chilling duration on the wake up time of *Aedes aegypti*. The reference temperature is 10°C.

**Tables S2.**

Fixed effects	Value	Std. Error	z-value	p-value
Intercept	0.8553	0.1417	6.037	1.57e-09
7 m per sec	-0.1622	0.1965	-0.826	0.4091
11 m per sec	-0.0573	0.1880	-0.305	0.7604
15 m per sec	-0.3171	0.1891	-1.677	0.0935
19 m per sec	0.0845	0.1914	0.442	0.6588

Fixed-effects coefficients of a mixed-effect binomial model of the impact of wind speed in the wind tunnel on the escape rate of *Aedes aegypti* measured in the IAEA reference flight test.

**Table S3.**

Comparison	Estimate	SE	df	t.ratio	p.value
Drone_100m_stationary vs Air 100m path	0.084	0.146	23	0.574	0.939
Drone_100m_stationary vs Drone_50m_stationary	0.270	0.364	23	0.741	0.880
Drone_100m_stationary – Ground	0.251	0.169	23	1.488	0.460
Drone_100m_path - Drone_50m_stationary	0.186	0.349	23	0.532	0.951
Drone_100m_path – Ground	0.168	0.135	23	1.242	0.607
Drone_50m_stationary – Ground	-0.018	0.359	23	-0.050	1.000

Comparison of the mortality rates of the different series in the field

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## Chapter 9

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# Conclusions

The overall aim of this research thesis was to help determine some key parameters with regard to the post-irradiation phase of mass rearing of sterile male mosquitoes, with a specific focus on their handling, transport and release, in the context of a SIT campaign as part of an AW-IPM programme. A range of optimal temperatures were developed for the storage and transport of male *Anopheles arabiensis*, *Aedes aegypti* and *Aedes albopictus*. The impact of compaction during storage was investigated and a maximum tolerable threshold determined. A standardised guide to mark all of the aforementioned species was developed and verified, in addition to being field tested on a large scale as part of an aerial release in Brazil. A novel and rapid flight test device which aims to assess male mosquito quality was devised, constructed and verified in rigorous laboratory experiments for male *Anopheles arabiensis*, *Aedes aegypti* and *Aedes albopictus*. The impact of varying environmental conditions relating to the time of day that sterile male releases could occur was investigated for both male *Anopheles arabiensis* and *Aedes aegypti*. Furthermore, as part of a USAID grant, an adult aerial release device was developed in conjunction with the NGO WeRobotics and field tested in Brazil in a series of MRR studies.

The topic of handling, transporting and releasing sterile male mosquitoes is vast and thus, this thesis could not address every parameter. It does however address and provide solutions to some of the most important aspects regarding the post-irradiation phases of mass rearing sterile male mosquitoes for release. Of note, a flight cylinder to assess the quality of both sterile male *Aedes* and *Anopheles* mosquitoes, was designed and verified. Prior to this, there was no tool available that allowed for a rapid assessment of sterile male mosquito quality. It is hoped that this quality control tool will become widely used and of great value in programmes conducting research or

commencing pilot SIT programmes against the main vectors of mosquito-borne diseases such as *Aedes aegypti*, *Aedes albopictus* and *Anopheles arabiensis*. It also has the potential, once tested, be used against various other species of mosquitoes which may become the focus of mosquito SIT pilot programmes.

Another key area which this thesis addresses is the aerial release by drone of sterile male mosquitoes. Although not part of the original research plan, it ended up becoming my fairy tale ending for this thesis. To the best of my knowledge, we were the first to release sterile male mosquitoes by drone as part of a mosquito MRR study anywhere in the world. With extremely promising results gained, it opens up the possibility of using drones to aerially release sterile male mosquitoes in other pilot projects or even a larger operational scale.

Although there are still many outstanding questions to be addressed in order to fully develop the mosquito SIT package before it can become fully operational, such as the severe bottleneck of a lack of GSS for several of the main mosquito disease vectors, several gaps in the literature have been addressed. With a warming climate aiding the most dangerous mosquito disease vectors to expand their current range and in turn placing a greater proportion of the global population into new disease endemic areas, time is of the essence with regard to developing complementary vector control tools such as SIT. Recent developments such as the collaboration of the WHO together with the FAO/IAEA to provide a guidance document governing all aspects of SIT for *Aedes* vectors highlights just how far the technique has come in just a few decades and how much closer it is to becoming an important part of the vector control “toolbox”. The research undertaken for this Doctorate has, and will continue to, aid countless member

states of the IAEA in their quest to develop the SIT package and begin the operational phase of releases in the coming years, in the fight against mosquito borne diseases and ultimately, the world's deadliest animal.